BIOMATERIAL AND CELL BASED CARTILAGE REPAIR STRATEGIES

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BIOMATERIAL AND CELL BASED CARTILAGE REPAIR STRATEGIES

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Chapter 1

General introduction, aims and contents of this dissertation

CLINICAL PROBLEMS

Injuries to human native cartilage tissue are particularly troublesome because cartilage has little to no ability to heal or regenerate itself. The reconstruction, repair, and regeneration of cartilage tissue continue to be one of the greatest clinical challenges, especially in orthopaedic and plastic surgery.

In orthopaedics, articular cartilage defects due to either acute destruction or chronic degeneration are commonly encountered in clinical practice but still represent a treatment challenge with unsatisfactory long-term results. These joint injuries not only limit physical activity and mobility of those afflicted, but the inability to move freely can cause significantly negative emotional and psychological impacts when individuals have to depend on family and healthcare providers for constant assistance to perform daily life functions. Also all kinds of joint defects are associated with an extremely high economic burden, which is largely attributable to the effects of disability, comorbid disease, and the expense of treatment[1]. Hence, successful restoration of the entire joint including the articular surface, and therefore, joint function, will be undoubtedly benefit numerous individuals, families and the whole society. At the moment, novel strategies and techniques for articular repair and regeneration are urgently needed.

Likewise, in the plastic and reconstructive surgery field, microtia-atresia, anotia, trauma, infections, or neoplasms of the intricately shaped external auricular cartilaginous structures are conditions that may require multiple surgical procedures for reconstruction[2]. Although the physiologic effects of ear deformity are negligible, the aesthetic and psychological impact on the patient can be profound. Significant ear malformations are prevalent in today's society affecting more than 5 percent of the population[3]. Reconstructing, replacing, or restoring auricular cartilage after congenital absence or traumatic loss continues to be a significant challenge for reconstructive surgeons.

CURRENT THERAPY AND ITS LIMITATIONS

Orthopaedic surgery

Unlike bone and other vascular tissues comprising the joint, cartilage is avascular and possesses limited capacity for repair and self-regeneration. Consequently, injury to cartilage in the articulating joints from trauma results in scar formation and possible arthritic changes that can lead to pain, stiffness, and loss of structure and function[4]. For these reasons, more than one million surgical procedures are performed in the United States each year involving cartilage replacement[5]. Cartilage grafting techniques have several disadvantages including the necessity for an open procedure, donor site morbidity, and problems associated with graft shaping, sculpting, and resorption[6]. In cases where there is insufficient autologous donor tissue, surgeons must rely on allogeneic[7] or artificial alloplastic implants. Allogeneic cartilage grafts have had unreliable clinical outcomes and carry increased risk of infection and disease transmission[8]. In anatomical areas where there is motion, alloplastic implants have a limited life span and can undergo migration and extrusion[9].

Lesions in the joint surface are commonly treated with microfracture[10], autologous cell implantation (ACI)[11], or osteoarticular autograft transfer system (OATS)[12]. Microfracture through the subchondral bone stimulates bleeding from the bone marrow permitting marrow cells to populate the defect. These putative chondrogenic precursor cells are stabilized by the blood clot that forms and produce a fibrocartilage matrix during the repair process[10]. The OATS procedure involves harvesting osteochondral plugs from non-weight-bearing areas and transfer to the defect. Although the plugs restore the cartilage surface, there may remain irregularities in the lesion due to plug placement. Many investigators have reported on clinical data on the re-implantation of free-floating autologous chondrocytes (ACI) into cartilage defects under a patch of periosteum or a collagen membrane to repair lesions in the articulating surface of knee joints[11, 13-15]. ACI has been in clinical use in human patients over two decades and has been performed on a large amount of patients worldwide. ACI has significantly reduced pain in patients – even the production of durable

cartilage-like tissue has been observed. The results after 3–9 years are very encouraging[16, 17]. However, this procedure still has some room for improvement, and the clinical assessment is somehow controversial. Confining the chondrocyte-saline suspension in the defect using a sutured periosteal flap or membrane is tedious and unpredictable, and the cells may be lost if the flap delaminates or is not well sealed.

Although patients have symptomatic relief and a few studies have showed decent histological assessment[18], there is still not enough definitive histological or biochemical data to support the contention that the new tissue that forms is characteristic of normal hyaline cartilage found on the joint surface comprised predominantly of type II collagen. ACI and microfracture most often result fibrous cartilage repair that is high in type I collagen and not durable in weight-bearing positions over the long term. Roberts *et al*. have reported that as many as 65 percent of second look biopsies showed fibrocartilage[19]. More recent modifications of the ACI technique being tested in Europe involve an open weave or spongeous matrix (MACI), frequently made from collagen or hyaluronic acid, where the cells are absorbed into the matrix before being secured in the lesion[20]. These woven type scaffolds do not provide any immediate biomechanical integrity and can be crushed by the forces placed on the joint. Furthermore, recent results suggest that the new tissue formed is also fibrocartilage and the fate of the cells is unknown[21, 22]. The long-term results using MACI techniques are not yet available.

Another problem of many current articular surface repair strategies is the failure of the repair tissue to integrate with the native cartilage surrounding the defect. As a result, fissures occur at the interface of the repair tissue and the existing host cartilage surrounding the lesion. Continued mechanical stress on the poor fibrocartilage matrix and weak interfaces can lead to breakdown of the scar tissue in the long term. A strategy that can generate durable hyaline articular cartilage, which will be predominantly type II collagen, and is capable of integrating with the surrounding cartilage matrix (without fissures) could improve the long-term outcome of joint surface repair. Regenerative medicine approaches involving biomimetic hydrogel scaffolds seeded with autologous cells could provide three-dimensional environments favorable for promoting chondrogenesis for joint surface repair.

Plastic and reconstructive surgery

Just like challenges in orthopaedics, cartilage regeneration has also been one of the primary goals for plastic surgeons. Instead of articular cartilage, plastic surgeons are most interested in restoring the craniofacial cartilage structures such as the nose and ear. Since Gillies carried out auricular reconstruction using autologous costal cartilage nearly a century ago, this approach has become the predominant surgical technique for partial and total reconstruction of the external ear[23]. Although this approach uses autologous tissue and can provide somewhat favorable results, there are several major disadvantages to this technique. There can be significant morbidity to the donor rib site such as pain, permanent deformity, and scarring[6]. There also can be operative complications such as an increased risk of pneumonia[24], pneumothorax, and atelectasis[25]. Furthermore, these costal cartilage grafts are very stiff and inflexible, and the cosmetic result is highly dependent on the surgeons' sculpting abilities and implantation techniques. Lastly, costal cartilage grafts can be subject to absorption[26] and progressive calcification[27] over time.

Numerous different kinds of alloplastic scaffold materials have been tried as framework substitutes for ear reconstruction by research laboratories, most of which have failed to enter the marketplace. Commercially, porous polyethylene (Medpor®) is one of the few alloplastic materials that are being used with some success clinically for craniofacial reconstruction and augmentation. Porous polyethylene, like costal cartilage grafts, is very stiff and inflexible. Although Medpor® implants have provided satisfactory cosmetic results in many craniofacial areas, it has limited utility for external ear reconstruction due to a high possibility of skin perforation and infection[28], especially if the Medpor® auricle is subjected to blunt trauma. Once protrusion through the skin occurs, the lesions do not heal and tend to result in breakdown of the tissue coverage. For these reasons, Medpor® is not an ideal substitute for reconstructing the human external auricle. Overall, novel strategies and techniques for ear reconstruction are being sought.

TISSUE ENGINEERING STRATEGY

The concept of tissue engineering presents a very promising alternative for generating tissues and organs in regenerative medicine. Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ[5]. This principle applies to cartilage repair and regeneration as well. However, only limited translation to clinics applying tissue engineering techniques has been achieved over the past two decades. Two of the most important components for achieving successful tissue regeneration are identifying sources of differentiated (chondrogenic) cells and suitable scaffold materials for cell delivery. These two critical aspects of tissue engineering cartilage are the focus of the studies presented in this dissertation.

Cell sources for cartilage regeneration

Cartilage has a low capacity for self-repair and, as a consequence, is highly susceptible to trauma or degenerative diseases such as osteoarthritis. Thus, the development of cell-based therapies that repair focal defects in otherwise healthy cartilage is an important research goal[29]. Chondrocytes build the macromolecular framework of the cartilage extracellular matrix (ECM) from three distinct classes of macromolecules: collagens (type II collagen), proteoglycans (mainly aggrecan), and a variety of non-collagenous proteins. Cartilage ECM is continually remodeled as chondrocytes replace matrix macromolecules lost through degradation. ECM turnover depends on the ability of chondrocytes to detect alterations in the macromolecular composition and organization of the matrix, such as the presence of degraded macromolecules, and to respond by synthesizing appropriate types and amounts of new ECM components. It is known that mechanical loading of cartilage creates mechanical, electrical, and physicochemical signals that help to direct the anabolic and catabolic activity of chondrocytes. In addition, the ECM acts as a signal transducer for chondrocytes. A prolonged and severe decrease in the use of the joint leads to alterations in the composition of the ECM and eventually to a loss of tissue structure and its specific biomechanical properties; whereas, normal physical strain stimulates the biosynthetic activity of chondrocytes and possibly internal tissue remodeling[16]. Numerous studies on engineering cartilage utilizing chondrocytes from juvenile animal sources have been reported. However, there are many unknown aspects of engineering cartilage using human chondrocytes — especially from middle-aged or elderly adults — which are critical for clinical application of tissue engineering in the field of orthopaedic surgery[30]. Although one kind of juvenile articular cartilage allograft — DeNovo® NT Natural Tissue Graft (Zimmer) was commercialized recently[31], it has not been widely accepted by orthopaedic surgeons worldwide due to lack of clinical evidence on effectiveness[32]. In this dissertation, both juvenile animal chondrocytes and elderly human chondrocytes will be examined in an attempt to better understand the applicability of age as an influence on cellular processes in a hydrogel system for cartilage regeneration.

Although chondrocytes have been used as the primary source for cartilage engineering thus far, mesenchymal stem cells (MSCs) may have significant advantages and potential to be an alternative cell source. Stem cells are a self-renewing cell population that exhibits an ability to undergo multilineage differentiation[33]. They were eventually defined as multipotent and able to differentiate into osteoblasts, adipocytes, myoblasts and more importantly, chondrocytes that could be applied in engineering human cartilage tissue[34]. One of the greatest challenges for using MSCs to generate cartilage is directing the cells down the desired chondrogenic differentiation pathway[35] while prohibiting ossification and hypertrophic differentiation. Growth factors including transforming growth factor (TGF) family, bone morphogenetic protein (BMP) family and insulin-like growth factor (IGF) family have been extensively examined for inducing chondrogenesis of MSCs. However, the current optimal formulations of chondrogenic medium, which are actually empirical, still do not yet produce stable chondrocytes[36]. While exogenous recombinant growth factors have been shown to be useful in laboratory studies, most have not been approved by the U.S. Food and Drug Administration (FDA) for clinical use because of numerous biologic and economic issues[37].

Besides, MSCs may exhibit a hypertrophic phenotype under chondrogenic induction resulting in calcification after ectopic transplantation[38, 39].

Several studies have shown the possibility of improving chondrogenesis through the coculture of the stem cells with chondrocytes[39-46]. Cocultures of different cell sources are based on the idea that the multi-signal events *in vivo* cannot be perfectly mimicked by adding a limited variety of growth factors to a monoculture of which the optimal cocktail remains largely elusive. This problem can be circumvented by the introduction of another cell source in the culture[47]. Rather than introduce another cell source, it is possible that soluble factors produced by chondrocytes could be used to effect differentiation of MSCs. The studies presented in this dissertation will show the effect of chondrocyte-conditioned medium on chondrogenesis of MSCs instead of directly mixing the MSCs and chondrocytes together to avoid confounding the contribution to matrix formation between the two different cell populations. Actually in recent years, there has been evidence showing that chondrocytes are capable of producing numerous soluble growth factors and cytokines that are released into the medium[48]. Also the design of chondrocyte-conditioned medium studies could differentiate the leading role on inducing chondrogenesis between MSCs and chondrocytes, since several of the coculture studies have even claimed that increased cartilage formation in these cocultures is mainly due to a trophic role of the MSCs in stimulating chondrocyte proliferation and matrix deposition by the chondrocytes rather than the MSCs actively undergoing chondrogenic differentiation[49, 50]. The experimental details of this coculture/conditioned medium on improving chondrogensis of MSCs will be demonstrated later.

Besides of cell source, differentiation and environmental factors there clearly is a need for improved delivery method and scaffold properties where aspects such as initial implantation, mechanical stability and biological incorporation are of importance. Thus we focus on the hydrogel carrier beside cell sources as core of this dissertation.

Hydrogel scaffolds for cartilage regeneration

Hydrogels are polymeric materials distinguished by high water content and diverse physical properties. They can be engineered to resemble the extracellular environment of the body's tissues in ways that enable their use in medical implants, biosensors, and drug-delivery devices. Cell-compatible hydrogels are designed by using a strategy of coordinated control over physical properties and bioactivity to influence specific interactions with cellular systems, including spatial and temporal patterns of biochemical and biomechanical cues known to modulate cell behavior[51].

In their natural environment, chondrocytes are surrounded by an extracellular matrix composed largely of collagen, glycosaminoglycan, and water, much like a hydrogel. Providing a temporary protective three-dimensional biodegradable scaffold possessing immediate mechanical integrity to stabilize the cells and promote hyaline cartilage matrix formation in a defect could avoid many of the long-term problems from fibrocartilage formation often encountered when using the ACI, MACI, and microfracture techniques. The combined high water content and elasticity of hydrogel polymers simulate cartilage tissue properties making them promising candidate matrices in which to suspend chondrocytes for cartilage tissue engineering. Hydrogel encapsulation of chondrocytes has been shown to generate neotissue resembling the native cartilage morphologically, biochemically, and, most importantly, biomechanically. Potential advantages of using hydrogels to deliver chondrocytes include: 1) ease of placement through minimally invasive procedures (*e.g.,* arthroscopically); 2) guided and/or accelerated neotissue formation by incorporating growth promoting cues; 3) *in situ* integration to native tissues; and 4) restoration of function throughout the entire healing process. The cells can be suspended in a three-dimensional hydrogel scaffold to maintain a finite spatial distribution of the cells.

Hydrogels still have their own disadvantages, however. For example, as one of the most commonly used natural hydrogels, fibrin gel has prochondrogenic properties and rapid degradation. This rapid degradation is theoretically beneficial for *in vivo* studies, but results in inferior tissue *in vivo* and makes long-term *in vitro* studies difficult to conduct[52]. Our group has previously described the use of hydrogels, including fibrin gel, solubilized collagen, hyaluronic acid, and sodium alginate, to engineer neocartilage *in vivo*. However, compared to native articular cartilage, these hydrogels are

mechanically fragile and would therefore be unable to support physiological joint load before ECM deposition occurs[53].

An ideal hydrogel for cartilage tissue engineering should initially support the loads found either in the joint (articular cartilage) or subcutaneously (auricular cartilage) and gradually degrade, transferring the loads to the evolving cartilage matrix[54]. In this dissertation, two promising maneuvers will be presented to improve the hydrogels to be better "cell carriers" for cartilage regeneration. One is the application of photochemical crosslinking techniques and the other one is the application of nondegradable/degradable hydrogel composites. Traditional chemical crosslinking and physical crosslinking are uncontrolled, time-consuming processes. Photochemical crosslinking is able to exert spatiotemporal control over the crosslinking process and thus can initiate the crosslinking process by irradiating with the light source, while the photosensitizer can be supplemented to the polymer mixture to induce the light's function[55]. Based on these discoveries, it is believed that photochemically crosslinking hydrogels enhances the physical parameters of the gel, while still permitting new cartilage formation both *in vitro* and *in vivo*. Another strategy relies on making nondegradable/degradable hydrogel composites to provide an adequate structural architecture similar to that surrounding the cartilaginous lesion. As such, the newly implanted hydrogels may require an additional nondegradable matrix to support the biomechanical forces distributed throughout the articular joint. Based on the findings, nondegradable/degradable hydrogel composites could provide additional structural support for mechanically weak hydrogels.

AIMS OF THIS DISSERTATION

The first aim of this dissertation was to evaluate different cell sources for the regeneration of cartilage. The cell types tested include chondrocytes and mesenchymal stem cells (MSCs) combined with biomimetic scaffold materials.

Rationales: Chondrocytes and mesenchymal stem cells have shown great potential for cartilage regeneration in both research and clinical fields. Since ACI has provided extensive proof demonstrating the feasibility and applicability of using autologous chondrocytes for cartilage repair, the combination of autologous chondrocytes, especially from elderly human source, with hydrogel system would be a realistic and meaningful strategy for cartilage tissue engineering. Since growth factors cause extensive concerns and most of them haven't been approved for clinical use by FDA, and chondrocytes are capable of producing numerous soluble growth factors and cytokines that are released into the medium, the coculture/conditioned medium strategy would be an efficient way to induce chondrogenesis of MSCs for cartilage repair and regeneration.

To address this goal, two specific aims were defined:

Specific aim 1: To evaluate the feasibility of cartilage matrix generation using elderly human chondrocytes seeded in a biomimetic hydrogel (Chapter 2).

Specific aim 2: To evaluate the feasibility of cartilage matrix generation using sheep chondrocytes; and, more importantly, using sheep bone marrow-derived mesenchymal stem cells (BMSCs) grown in conditioned media collected from chondrocyte cultures combined with biomimetic scaffolds (Chapter 3-4).

The second aim of this dissertation was to explore the novel maneuvers to improve multiple kinds of biomimetic hydrogels as "cell carriers" for cartilage generation.

Rationales: Hydrogels have shown to be one of the optimal candidates for biomimetic scaffold materials for cartilage regeneration. However, most of the traditional natural hydrogels have proved to be mechanically weak and couldn't withstand the intense pressure either in the joint or subcutaneously. Photochemical crosslinking and the combination of nondegradable/degradable hydrogel composites could enhance the mechanical properties theoretically. We presumed that those

two novel strategies applying on suitable hydrogels would be able to strengthen the scaffolds, while maintaining favorable cell viability and neocartilage ECM formation both *in vitro* and *in vivo*.

To address this goal, two specific aims were defined:

Specific aim 1: To evaluate the feasibility of cartilage generation using photochemically crosslinked hydrogels (Chapter 5-6).

Specific aim 2: To test nondegradable/degradable hydrogel composites as scaffold materials for cartilage generation (Chapter 7-8).

OUTLINE OF THIS DISSERTATION

Based on the aims of this dissertation outlined above, it is constituted of two major components. One focuses on evaluation of "cell sources" and the other one focuses on "hydrogel scaffolds" – two of the mainstays for regenerating cartilage applying tissue engineering and regenerative medicine strategies.

The topic of Chapters 2-4 is "evaluation of different cell sources for cartilage regeneration". Juvenile chondrocytes have been commonly used for cartilage tissue engineering studies by researchers in the field. However, whether the scientific results achieved based on juvenile animal chondrocytes could be also translated into the applications of human chondrocytes still remains unclear. Especially in orthopaedic surgery, most patients suffering from articular cartilage defect in clinical world are middle-aged or older; thus, the study of chondrocytes harvested from this patient population to engineer cartilage would be necessary. Chapter 2 therefore, describes the feasibility of neocartilage regeneration using elderly human articular chondrocytes (obtained from 50-60 years old donors) encapsulated in fibrin hydrogel. However, since only limited amounts of autologous donor cells are available, mesenchymal stem cells (MSCs) could provide a more extensive pool of regenerative cells and have a great potential to significantly alter the applications of tissue engineering. Chapter 3 and 4 describe the feasibility of neocartilage regeneration using both chondrocytes and BMSCs from sheep and a novel fibrous collagen scaffold. Chapter 3 firstly proved the feasibility of neocartilage regeneration using juvenile sheep chondrocytes (3-6 months old) seeded on this collagen scaffold, and Chapter 4 describes the feasibility of cartilage regeneration using BMSCs on the same collagen scaffold. In order to prevent calcification and to induce better chondrogenic differentiation, our group introduced conditioned media collected from sheep chondrocyte cultures to grow BMSCs, which could be a more realistic and efficient way for chondrogenesis and future clinical application of cartilage tissue engineering.

The topic of Chapters 5-8 is "exploration of the novel maneuvers to improve multiple kinds of biomimetic hydrogels as "cell carriers" for cartilage regeneration". Chapters 5 and 6 describe the application of photochemically crosslinked hydrogels for neocartilage regeneration. Chapter 5 is focused on the studies of the properties and the evaluation of various parameters of dual crosslinked (photochemical plus chemical) collagen gel as suitable scaffold to delivery chondrocytes *in vitro*. Chapter 6 describes that a novel photochemically crosslinked gelatin-methacrylamide (gelMA) hydrogel scaffold provides a favorable microenvironment for both articular and auricular chondrocytes to produce extracellular matrix *in vivo*. Chapter 7-8 describe the application of nondegradable/degradable hydrogel composites for cartilage regeneration. Chapter 7 is focused on the evaluation of novel porous poly(vinyl alcohol (PVA)/fibrin gel composites, and Chapter 8 describes the application of PEG-LA-DM/PEGDM copolymer hydrogel composites for neocartilage regeneration. Swine articular chondrocytes have been used in all of the studies in this part of work.

Chapter 9 is the discussion, conclusions, and the future perspectives.

Chapter 2

Properties of cartilage engineered from elderly human chondrocytes for articular surface repair

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ABSTRACT

Numerous studies on engineering cartilage utilizing chondrocytes from juvenile animal sources have been reported. However, there are many unknown aspects of engineering cartilage using human chondrocytes — especially from middle-aged or elderly adults — which are critical for clinical application of tissue engineering in the field of orthopaedic surgery. The primary aim of this study was to engineer neocartilage tissue from 50–60-year-old human chondrocytes in comparison to engineered cartilage made from juvenile swine chondrocytes (JSCs). Articular chondrocytes from middle-aged, nonarthritic humans and juvenile swine were isolated and placed in culture for expansion. The chondrocytes (passage 1) were mixed in fibrin gel at 40–60 X 10^6 cells/mL until polymerization. Cells/nodule constructs and devitalized cartilage–cells/hydrogel–devitalized cartilage constructs (three-layered model) were implanted into subcutaneous pockets of nude mice for 12, 18, and 24 weeks. The specimens were evaluated histologically, biochemically, and biomechanically. This allowed for direct comparison of the cartilage engineered from human versus swine cells. Histological analysis demonstrated that samples engineered utilizing chondrocytes from middle-aged adults accumulated basophilic, sulfated glycosaminoglycans (sGAG), and abundant type II collagen around the cells in a manner similar to that seen in samples engineered using JSCs at all time points. Biochemical analysis revealed that samples made with human cells had about 40%–60% of the amount hydroxyproline of native human cartilage, a trend parallel to that observed in the specimens made with swine chondrocytes. The amount of sGAG in the human chondrocyte specimens was about one-and-a-half times the amount in native human cartilage, whereas the amount in the samples made with swine chondrocytes was always less than native cartilage. The biomechanical analysis revealed that the stiffness and tensile of samples made with human cells were in a pattern similar to that seen with swine chondrocytes. This study demonstrates that chondrogenesis using articular chondrocytes from middle-aged adults can be achieved in a predictable and reliable manner similar to that shown in studies using cells from juvenile animals and can form the basis of engineering cartilage with degradable scaffolds in this patient population.

INTRODUCTION

Following trauma to the articulating joint surface, cartilage has limited innate capability for healing and self-regeneration. Consequently, injury to cartilage can result in arthritic changes that can lead to pain, stiffness, and loss of structure and function[4]. Common surgical treatments currently employed include microfracture, autologous chondrocyte implantation (ACI), and osteoarticular autograft transfer system (OATS). The microfracture technique relies on the random spontaneous transformation of bone marrow cells into a chondrogenic lineage, which is often incomplete and inefficient. Both ACI and OATS require cartilage harvesting techniques that can cause additional morbidity to uninvolved areas of the already injured joint. ACI involves performing an arthrotomy and placement of culture expanded autologous chondrocytes suspended in saline into the articular defect and covering with a periosteal flap or collagen membrane. Clinical experience has shown that there is considerable morbidity and prolonged recovery associated with the ACI procedure. Further, placement of the flap is tedious and has unpredictable results. All these surgical approaches can provide temporary symptomatic relief, but the long-term outcomes are inconsistent in part, because the reparative tissue that forms does not resemble native cartilage morphologically, biochemically, or biomechanically. Therefore, developing more effective therapies, while at the same time decreasing morbidity, is the overarching clinical goal. The field of cartilage tissue engineering has sought new approaches in which chondrocytes are transferred on biocompatible scaffolds to produce new extracellular matrix (ECM) for the repair of cartilage defects[56-58]. Numerous groups have developed methodologies to generate cartilage tissue through cell encapsulation into hydrogels or seeding them onto scaffolds. However, the vast majority of these studies have used cells from juvenile animal sources — cells that are believed to have a strong propensity to form new ECM. Our work with a variety of hydrogels demonstrates successful tissue engineering of cartilage using swine, rabbit, or sheep cells from healthy young animals for the evaluation of chondrogenesis[59, 60]. Although cartilage can be easily generated with chondrocytes from juvenile animals, these favorable results may not accurately model the clinical conditions of patients requiring treatment for articular cartilage lesions; many of the patients requiring chondral defect repairs are middle aged or older. Several studies have reported that articular chondrocytes from aged human and animal sources have an impaired ability to generate new ECM *in vitro*. Less is known, however, whether aged human chondrocytes can be reinvigorated to produce neocartilage much like that observed with juvenile animal chondrocytes. More importantly, this should be studied in comparative studies *in vivo*. Although studies utilizing juvenile animal cell sources provide fundamental information for the field of cartilage tissue engineering, further studies using human chondrocytes are required for the transition of bench top work into clinical trials. Building on a solid foundation of *in vitro* and *in vivo* animal studies, we hypothesized that human cartilage could be engineered with similar morphological, biochemical, and biomechanical properties as engineered cartilage generated by juvenile swine chondrocytes (JSCs). In this study, articular chondrocytes from middle-aged healthy, nonarthritic, human donors were encapsulated in fibrin gel (FG) and compared with articular JSCs. The aims of this study were (i) to determine whether middle-aged human articular chondrocytes encapsulated in hydrogel could form neocartilage, (ii) to evaluate whether the neocartilage could integrate with existing human cartilage, and (iii) to assess whether the tissue-engineered cartilage generated by middle-aged human chondrocytes was comparable to that generated by JSCs, morphologically, biochemically, and biomechanically.

MATERIALS AND METHODS

Cartilage harvest and cell isolation

All actions were approved by the Institutional Animal Care and Use Committee (IACUC) and IRB of the Massachusetts General Hospital. Human articular cartilage (age range: 50–60 years old, average age: 55 years old) was obtained from National Disease Research Interchange. Native cartilage

measuring 1 X 1 X 0.2 cm was collected from each sample for control assays. The remainder tissue was finely minced and digested using 0.1% collagenase type II (Worthington Biochemical, Lakewood, NJ) for 16 — 18 h at 37^0C. After digestion, the chondrocytes were rinsed and washed twice with PBS. Cell viability was assessed using the trypan blue dye exclusion. Cell isolations with > 90% viability were plated in 150 cm^2 culture flasks at an initial density of 6 X 10^3 cells/cm^2 and cultured in standard chondrocyte media consisting of Ham's F-12 medium with L-glutamine supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 50mg/mL ascorbic acid, 100U/mL penicillin, and 0.1mM nonessential amino acids at 37^0C in 5% CO2. Media were changed three times per week. The cells were removed from the flasks with 0.5% trypsin-EDTA (wt/vol) (Gibco, Carlsbad, CA) when they reached 90% confluence and washed twice with PBS. Articular cartilage was also harvested from the knees of 3-month-old swine and processed in the same fashion as human chondrocytes. Passage 1 (P1) cells were used for the construct assembly.

Construct assembly and implantation

Articular chondrocytes from both healthy human donors and young swine were suspended in a FG solution. To make the FG, bovine fibrinogen (Sigma, St. Louis, MO) was prepared in sterile saline at a concentration of 80 mg/mL[57]. Thrombin (GenTrac, Middleton, WI) was diluted to 50U/mL in saline. One hundred twenty-five microliters of cells in the fibrinogen solution and an equal volume of thrombin solution were placed into a bone wax well measuring 0.8 X 0.8 cm and mixed with the micropipette. Final nodule construct volume was 0.8 X 0.8 X 0.35 cm. Based on our previous experience, the cell concentration of swine chondrocytes was 40 X 10^6/mL[57, 61, 62], and the human chondrocytes was 60 X 10^6/mL. To evaluate the integration of neocartilage with native articular cartilage, three-layered constructs were assembled as previously described[61]. The FG solution containing either human or swine chondrocytes was prepared in the same way as explained previously. Discs of human and swine cartilage were made with a 6-mm biopsy punch and devitalized by a previously documented procedure using five freeze-thaw cycles[63]. Subsequently, 100 mL of the cell/FG was placed on the top of one disc and a second disc was placed on the top of the cells in the gel, creating a cartilage–cell/ hydrogel–cartilage construct to determine cartilage integration. A 7–0 monofilament suture was used to secure the layers in place. After the *in vivo* period, the suture was removed and constructs were tested for integration. All constructs (nodule constructs, n = 60; three-layered constructs, n = 72) were implanted into subcutaneous pockets on the backs of nude mice (Massachusetts General Hospital). Samples were harvested at 12, 18, and 24 weeks.

Histological analysis

Nodules (n = 3) and three-layered constructs (n = 3) were randomly selected from each of the time points and were placed in 10% phosphate-buffered formalin for 24 h. They were subsequently embedded in paraffin and sectioned. Sections were prepared from a minimum of two areas from each specimen and were stained with hematoxylin and eosin (H&E) for evaluation of gross morphology of the neotissue. Other sections were stained with Safranin-O and Toluidine Blue to verify the production of sulfated glycosaminoglycans (sGAG). The sections were evaluated in a blinded manner by three independent reviewers, to determine the presence or absence of neocartilage formation.

Immunohistochemical analysis

Mouse anti-human collagen type I (COL I) antibody (Accurate Chemical & Scientific Corp., Westbury, NY) and mouse anti-human collagen type II (COL II) antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) were used. Prior to use, the antibody was diluted 1:1000 in 1% bovine serum albumin (Gibco) in PBS. For negative control, N-universal negative control (DAKO, Glostrup, Denmark) was used. The incubation time was 60 min. Sections were washed extensively in PBS and an appropriate secondary antibody (Dako) was used for 20 min, followed by wash in PBS (3 X 5 min). Detection of the immunoreaction was achieved by adding 3, three-dimensionaliaminobenzidine (Dako), which was used as substrate chromogen for 5 min. Slides were washed in distilled water and counterstained with hematoxylin to visualize the cell nuclei.

Biochemical analysis

Quantification of sGAG was determined with the dimethylene blue method and the hydroxyproline was determined using the chloramine-T method in the nodule constructs as previously described by our laboratory[62]. Biochemical analysis was performed on four to six samples per group at each time point. One 40–50 mg piece of engineered cartilage tissue was collected from each nodule specimen and those pieces were weighed and lyophilized for 24 h. Dehydrated specimens were digested with papain solution (125 μg/mL papain type III, 100mM phosphate, 10mM L-cysteine, and 10mM EDTA [pH 6.3]) at 60^0C for 16 h. Both sGAG and hydroxyproline content of the specimens were determined by using shark cartilage (Sigma) as a standard. Samples and standards were analyzed in duplicate and reported as a percentage of wet tissue weight as well as dry tissue weight.

Biomechanical analysis

Samples were frozen at - 80^0C immediately after harvest for biomechanical testing and thawed at room temperature in PBS with proteinase inhibitors immediately before testing, when the fibrotic capsule surrounding the constructs was removed.

Compression testing (nodule constructs).

Samples were cut into disks with a 6-mm diameter using a disposable biopsy punch and placed in a confining chamber that was mounted in an Enduratec EL2100 mechanical testing frame using a 10-N load cell (Bose, Eden Prarie, MN). Samples were equilibrated at room temperature in 0.15M PBS (pH 7.4) containing 100U/ mL penicillin G and 100mg/mL streptomycin. After mounting each disk in the confined compression chamber, the distance between the porous platen and the chamber was decreased until a signal of ~5 g (50 mN) was detected by the load cell. This distance was taken to be the sample thickness. Each disk was compressed by 10 sequential increments of 2.5%–3.0% static strain, up to a maximum of 25%–30% total strain. After each increment, the load was recorded every 0.5 s for 100 s. Stress relaxation data were fit to a poroelastic model of material behavior to calculate the equilibrium modulus[64].

Tensile testing (three-layered construct model).

The integration of engineered cartilage with native cartilage was assessed by pulling three-layered constructs to failure in tension, a technique described by our group in several studies over the last decade[61, 65, 66]. The flat ends of the constructs were attached to Plexiglas rods, using quick-setting cyanoacrylate glue. The rods were then mounted in the jaws of the Enduratec EL2100 mechanical testing frame. One milliliter of PBS was placed around the constructs to prevent sample dehydration. Tensile displacements were applied to the constructs at a rate of 4–10 μm/s, corresponding to a bulk strain rate of 0.15%/s for each sample, and the resultant load was recorded at a rate of 5 points/sec. Samples were pulled to failure in tension as indicated by either visible separation of cartilage disks or a measured load of < 0.05 N. Applied displacements and measured loads were normalized to sample thickness and area, respectively. Using these data, a stress–strain curve was constructed for each specimen. From the stress–strain curve, the ultimate tensile strength (σ_{UTS}) was determined by inspection as the highest stress supported by the sample, and the failure strain (ε_f) was determined by inspection as the strain at which further increases in strain produced lower stresses. The failure energy (E_f), defined as the area under the stress–strain curve until failure, was calculated by a Reimann sum method with the partition element given by the strain interval between data points. The dynamic tensile modulus (M) for the given strain rate of each sample was calculated as the slope of the linear portion of the stress–strain curve, using a standard least squares algorithm, with r^2 values > 0.9 as criteria for acceptable linearity.

Statistical analysis

All the statistical analysis was performed using two-way ANOVA to test the differences in mean value of GAG and hydroxyproline content as well as the biomechanical parameters of the neocartilage and three-layered construct model constructs over the three time points in this study. Mean values were reported with standard deviations. Significance was assigned at $p < 0.05$.

RESULTS

All samples were recovered from nude mice at 12, 18, and 24 weeks. For the nodule constructs, the initial shape was conserved over time in all the experimental groups. The specimens resembled cartilage in color and texture and were resistant to external compression. For the three-layered constructs, the native articular cartilage disks maintained their glistening white surface throughout the duration of implantation. Neotissue formation in the intradisk space ranged from pinkish white to pearlescent in all the experimental groups. Although the appearance of the neotissue was notable in the intradisk space, there were no obvious gaps between the new cartilage and the native cartilage disks (Fig. 1).

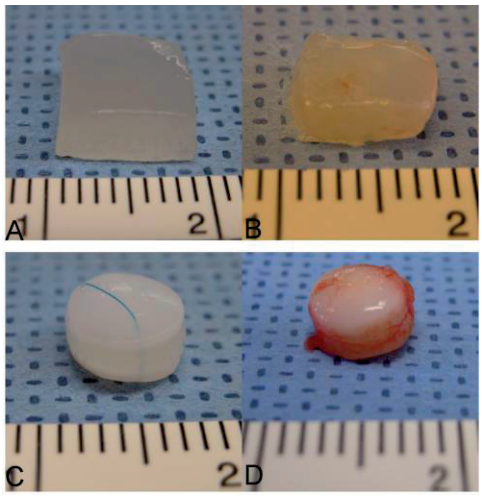

Figure 1. Gross appearance. Nodule construct made with elder human articular chondrocytes before (A) and after (B) 18 weeks *in vivo*. Three-layer construct made with devitalized human articular cartilage–human chondrocytes/ fibrin gel–devitalized human articular cartilage before (C) and after (D) 18 weeks *in vivo*.

Histological evaluation

For the nodule constructs H&E staining of samples made with chondrocytes from both middle-aged human sources and JSCs exhibited a similar histomorphology characteristic of cartilage (cells within basophilic ground substance) at all time points. The presence of sGAG was also evident with intense Safranin-O and Toluidine Blue staining at all time points from all the experimental groups (Fig. 2).

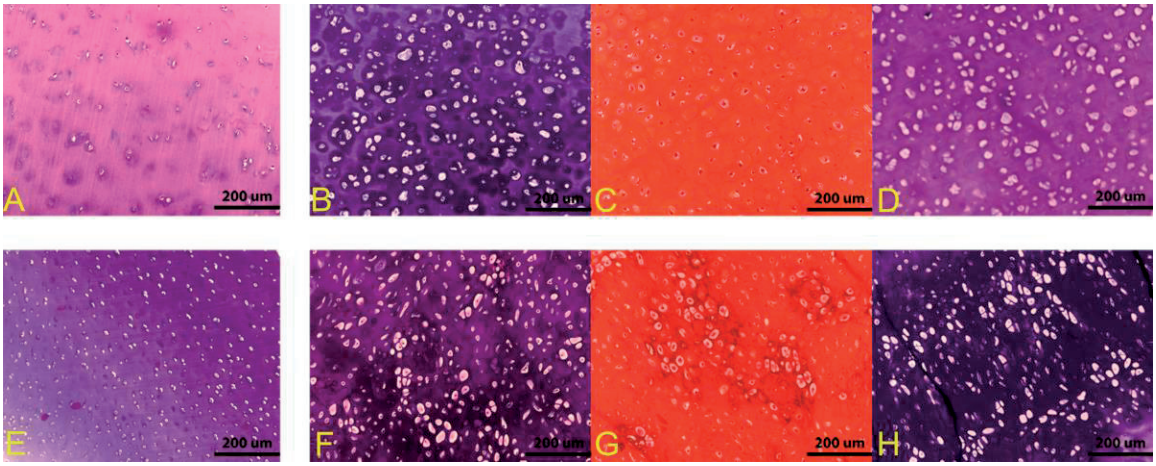

Figure 2. Histological evaluation of nodule samples (after 18 weeks *in vivo*). Samples made with elder human articular chondrocytes were stained with hematoxylin and eosin (H&E) (B), Safranin-O (C), and Toluidine Blue (D), comparing with human native cartilage stained with H&E (A) from the same donor. Samples made with

juvenile swine articular chondrocytes were stained with H&E (F), Safranin-O (G), and Toluidine Blue (H), comparing with swine native cartilage stained with H&E (E) from the same donor (magnification, 100 X).

For the three-layered constructs, H&E staining of samples made with native cartilage disks and chondrocytes from both middle-aged human sources and JSCs revealed similar new ECM formation of neocartilage tissue within all intradisk areas. Increased red staining with Safranin-O as well as increased blue staining with Toluidine Blue revealed the presence of abundant sGAG in the newly formed tissue. Staining was less intense in the native cartilage due to the freeze-thaw devitilization process used to make the nonviable native cartilage disks, which was consistent with the findings in our previous experiments[60, 61]. In all of the experimental groups, the new matrix formed between the native cartilage disks appeared to fill all surface irregularities and in multiple samples there was evidence of neocartilage penetrating into the adjacent cartilage disks. There were no gaps observed at the interfaces of the neotissue and the native cartilage disks (Fig. 3).

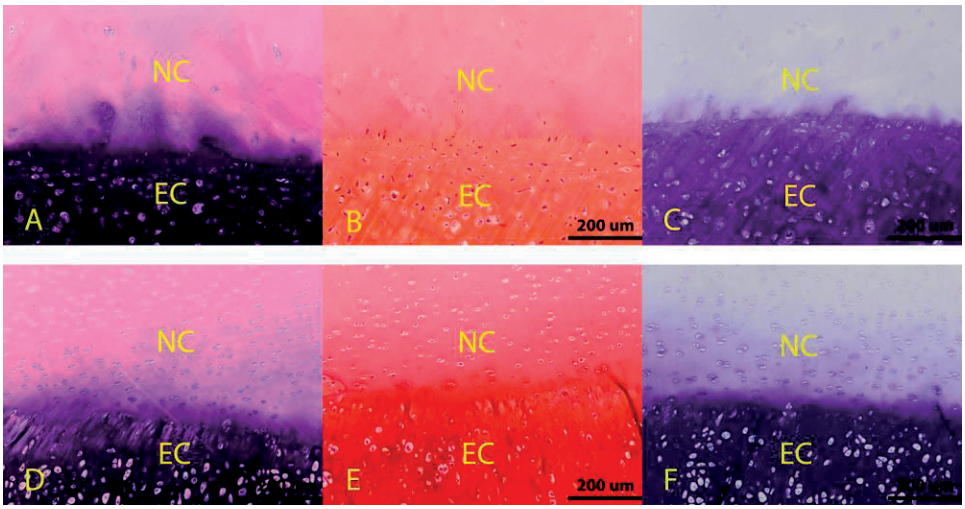

Figure 3. Histological evaluation of three-layer constructs (after 18 weeks *in vivo*). Human cell source samples were stained with H&E (A), Safranin-O (B), and Toluidine Blue (C). Swine cell source samples were stained with H&E (D), Safranin-O (E), and Toluidine Blue (F) (magnification, 100 X; NC, native cartilage; EC, engineered cartilage).

Immunohistochemical staining

Immunostaining for COL I was negligible in both the nodule constructs and the three-layered constructs. By contrast, immunostaining for type II collagen demonstrated uniform staining throughout the new ECM on the nodule constructs. The three-layered constructs also showed the presence of type II collagen in both the new cartilage matrix and the native cartilage disks (Fig. 4).

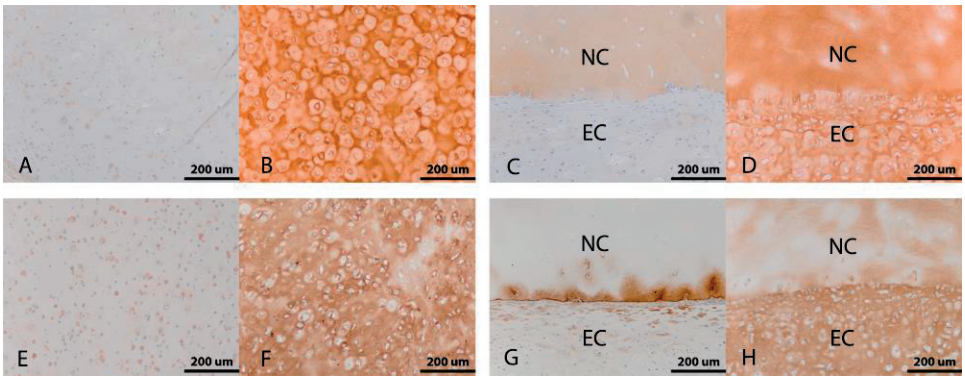

Figure 4. Immunohistochemical evaluation of nodule and three-layer samples (after 18 weeks *in vivo*). Nodule constructs made with elder human articular chondrocytes were stained for type I (A) and type II collagen (B), and human cell source sandwich samples were stained for type I (C) and type II collagen (D). Nodule constructs made with juvenile swine articular chondrocytes were stained with type I (E) and type II collagen (F), and swine

cell source sandwich samples were stained with type I (G) and type II collagen (H) (magnification, 100 X; NC, native cartilage; EC, engineered cartilage).

Biochemical analysis

GAG content

The amount of GAG in the native JSCs was 453 ± 32 µg/mg dry tissue (Fig. 5). However, the amount of GAG measured in the native human cartilage (211 – 31 mg/mg dry tissue) was less than half of that measured in the native swine tissue. At the 12-week time point, the amount of GAG in the swine chondrocyte nodules was 264 – 12 mg/mg dry tissue, or slightly more than half of the native swine cartilage, whereas the amount of GAG in the nodules made with human chondrocytes was 326 – 26 mg/mg dry tissue — about 154% of the amount measured in the native human tissue. A similar result was observed for the human chondrocyte nodules at 18 weeks, but the amount in the swine chondrocyte constructs at 18 and 24 weeks was only about 11%–15% of the amount measured in the native swine.

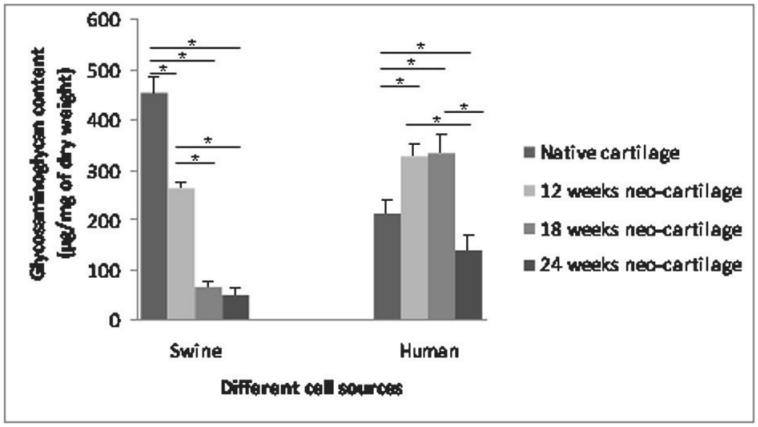

Figure 5. Glycosaminoglcyan (GAG) contents are shown in both wet weight and dry weight (n = 4–5). There was statistically significant difference in the amounts of GAG between tissue-engineered cartilage and corresponding native cartilage at all the time points ($p < 0.05$). For swine source samples, there was no statistically significant difference in the amounts of GAG between specimens harvested at 18 and 24 weeks ($p > 0.05$); however, both of which had a statistically significant difference comparing with the specimens harvested at 12 weeks ($p < 0.05$). For human source samples, there was no statistically significant difference in the amounts of GAG between specimens harvested at 12 and 18 weeks ($p > 0.05$); however, both of which had a statistically significant difference comparing with the specimens harvested at 24 weeks ($p < 0.05$). (*Indicates statistically significant difference; $p < 0.05$).

Hydroxyproline content

The amount of hydroxyproline in the native human tissue was 39 ± 10 µg/mg dry tissue and that in the native swine tissue was 35± 5 µg/mg dry tissue. These initial amounts were not statistically different from each other ($p > 0.05$). The amount of hydroxyproline in the nodule specimens made with middle-aged human articular chondrocytes at 12, 18, and 24 weeks was 19 ± 4, 15 ± 6, and 25 ± 2 µg/mg dry tissue, respectively. By comparison, the amount of hydroxyproline in the nodule specimens made with articular JSCs at 12, 18, and 24 weeks was 18 ± 2, 16 ± 4, and 29 ± 1 µg/mg dry tissue, respectively. There were no statistical differences between the amounts of hydroxyproline for human chondrocytes versus swine chondrocytes at each time point. However, the amount of collagen in the test specimens at each time point for both human and swine chondrocytes was statistically different from the level in the native cartilage ($p < 0.05$) (Fig. 6).

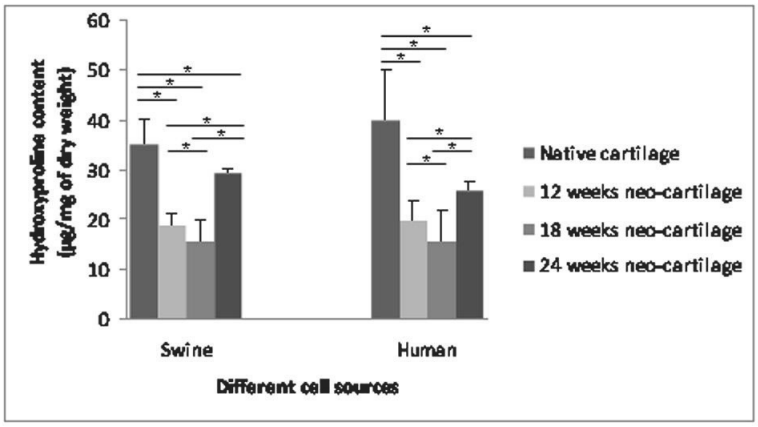

Figure 6. Hydroxyproline contents are shown in both wet weight and dry weight (n = 4–5). There was statistically significant difference in the amounts of hydroxyproline between tissue-engineered cartilage and corresponding native cartilage at all the time points ($p < 0.05$). There was also statistically significant difference in the amounts of hydroxyproline among the three experimental groups harvested at 12, 18, and 24 weeks ($p < 0.05$) for both swine and human source samples. (*Indicates statistically significant difference; $p < 0.05$).

Biomechanical analysis

Equilibrium modulus (applied to nodule constructs)

The equilibrium modulus of cartilage nodule samples grown from human and swine chondrocytes showed similar values at 12 and 18 weeks (Fig. 7). However, both the human and swine samples experienced a small drop in modulus at 24 weeks. It should be noted that the modulus of the cartilage with either JSCs or human cells never exceeded 10% of the modulus of native cartilage (juvenile swine cartilage: 457 ± 11 kPa; human native cartilage: 332 ± 12 kPa). The equilibrium modulus of samples grown from human and swine chondrocytes differed by 10%–40% across the time of the study, with no statistical difference between the two cell types at any time point.

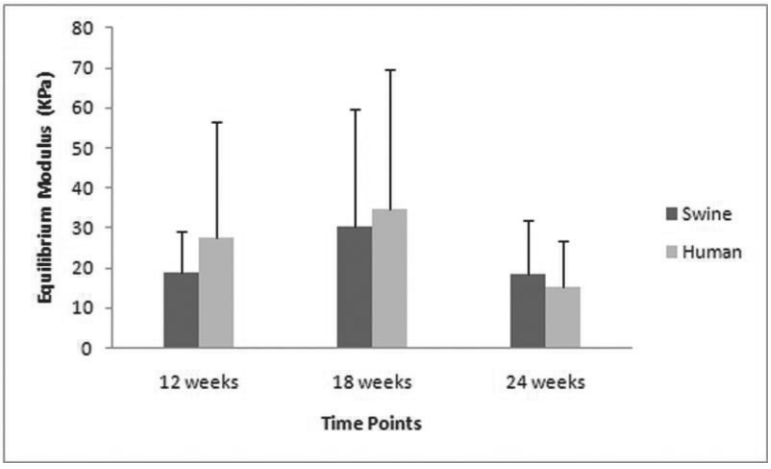

Figure 7. Equilibrium modulus (n = 8–12). There was no statistically significant difference in the equilibrium modulus between the human chondrocyte and swine chondrocyte groups at each time point ($p > 0.05$).

Tensile properties (applied to three-layered constructs)

The tensile properties of three-layered samples made with swine chondrocytes differed from those made with human chondrocytes over time (Fig. 8). The UTS and the tensile modulus made from JSCs increased over time and were consistently higher than those with human cells, with greater than a three-fold difference in UTS at 24 weeks ($p < 0.05$) and more than a seven-fold difference in tensile modulus at 24 weeks ($p < 0.05$). The UTS and tensile modulus of the human chondrocyte samples decreased from 18 to 24 weeks.

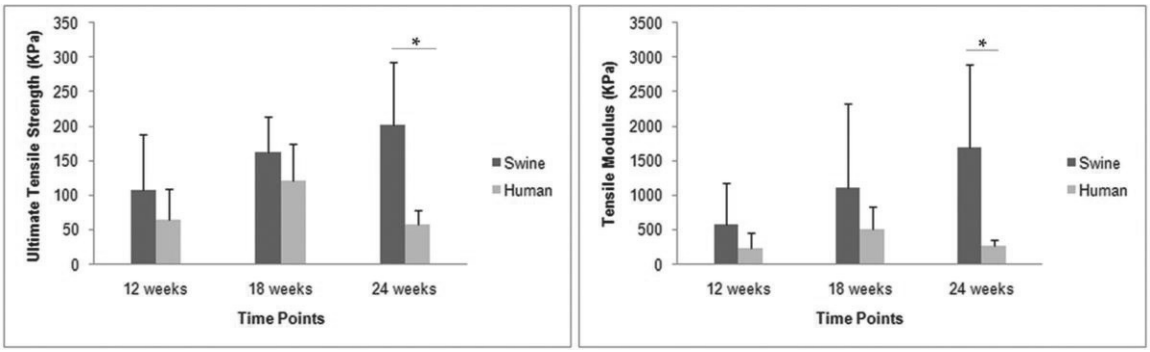

Figure 8. Ultimate tensile strength and tensile modulus (n = 8–12). There was no statistically significant difference between human chondrocyte and swine chondrocyte groups at both 12 and 18 weeks ($p > 0.05$). However, there was statistically significant difference at 24 weeks ($p < 0.05$). (*Indicates statistically significant difference; $p < 0.05$).

In contrast to the trends seen in tensile strength and modulus data, the failure strain was higher in samples made from adult human cells, with a 90% difference measured at 24 weeks. The failure energy of samples made from human and swine cells was similar at 12 weeks, but was ~ 40% higher in swine samples at 18 weeks and more than two-fold higher at 24 weeks ($p < 0.05$) (Fig. 9).

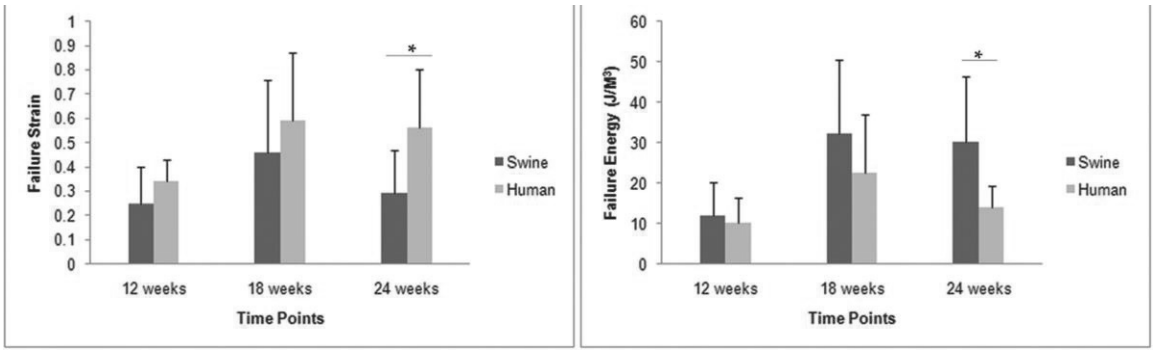

Figure 9. Failure modulus and failure energy (n = 8–12). There was no statistically significant difference between human chondrocyte and swine chondrocyte groups at both 12 and 18 weeks ($p > 0.05$). However, there was statistically significant difference at 24 weeks ($p < 0.05$). (*Indicates statistically significant difference; $p < 0.05$).

DISCUSSION

Most of the published work on engineering cartilage on biodegradable scaffolds, both *in vitro* and *in vivo*, has used juvenile animal chondrocytes. This may be due to the ease of acquiring large numbers of chondrocytes from young animals for experimentation and the difficulty in obtaining sufficient amounts of healthy human tissue. Our own studies have predominantly employed JSCs to generate cartilage *in vivo* in nude mice or in situ in young adult animals[59, 61, 62, 67]. Nonetheless, these studies have built a solid foundation of experimental animal data demonstrating that the concepts of tissue engineering can be employed to produce new cartilage tissue that can potentially heal defects in the articular surface of joints.

ACI has established that chondrocytes can be harvested, expanded in culture, and implanted into cartilage defects to stimulate a reparative process[68, 69]. Previous studies have suggested, however, that articular chondrocytes isolated from aged human and animal cartilage sources could have an impaired capacity to produce ECM[70-73]. This is crucial since patients sustaining injuries may more

likely be middle aged or older. Far less is known about engineering cartilage for joint surface regeneration in these age groups, which makes the study of chondrocytes from middle-aged humans necessary and clinically relevant.

Our team has extensive experience using different hydrogels for encapsulating animal chondrocytes to generate ECM for possible knee cartilage repair[59, 74-77]. While it is well established that human chondrocytes will make new ECM, few studies have made a direct comparison between human and animal chondrocytes to engineer neocartilage tissue[78]. In this study, we made a direct comparison between the ECM generated by JSCs and that made by chondrocytes from middle-aged human sources when both were encapsulated in the same fashion in a FG polymer. The results indicate that articular chondrocytes from middle-aged human donors do, in fact, have the capacity to generate tissue-engineered cartilage *in vivo* when encapsulated in this hydrogel scaffold. Generally, the engineered neocartilage produced with articular chondrocytes from these middle-aged human donors has histological, biochemical, and biomechanical characteristics similar to that of neocartilage made with articular chondrocytes from JSC donors. Thus, the data generated from this study suggest that it would be possible to use autologous articular chondrocytes to engineer neocartilage for joint resurfacing and repair in the middle-aged population.

The histological and immunohistochemical results in constructs made with human chondrocytes were indistinguishable from those using JSCs. The histological staining of the ECM was uniform across the specimens in both groups and the Safranin-O images demonstrated the production of sGAG typically observed in native cartilage. COL II, a significant component of the ECM of hyaline articular cartilage, defines the biomechanical properties of this tissue. The immunostaining of the native cartilage from both groups showed intense staining for COL II and faint-to-negligible staining for COL I as would be expected. The engineered cartilage specimens from both groups showed similar staining patterns indicating the production of COL II and little staining for COL I. There were no observable differences between the engineered specimens made with JSCs and those made with middle-aged human chondrocytes.

In addition to the histology, the biochemical analysis revealed that the encapsulated cells were generating GAG, particularly in the early phase of *in vivo* incubation. The amount of GAG measured in the native JSCs was nearly twice that measured in the native human cartilage. The samples made with JSCs demonstrated moderate amounts of GAG in the 12-week samples—about half that of the native cartilage. For unexplained reasons, the swine specimens harvested at 18 and 24 weeks had much less GAG. By contrast, in the groups of specimens made with middle-aged human chondrocytes, the amount of sGAG was about 30%– 40% higher than native cartilage at 12 and 18 weeks. This suggests that these aged cells liberated from their native matrix can be reinvigorated to produce large amounts of GAG as has been reported previously in chondrocytes from old-aged sheep[79]. The amount of GAG measured at 24 weeks remained about 75% of native human cartilage. These results indicated that the middle-aged human chondrocytes encapsulated in FG polymer retained a strong ability to produce high amounts of GAG. A drop in the amount of GAG was noted in the later time points in constructs from both cell sources. This decline may be due to the lack of biomechanical forces in this subcutaneous implant model. This decline was noted by week 18 in the samples made with juvenile swine cells, whereas the constructs made with human cells did not decline until the 24 week specimens. These results may be due to inherent differences between the species or the different ages of human versus the swine cells used in this experiment.

Whereas the GAG content of native swine cartilage was nearly double that of native human cartilage, the amount of collagen in the human cartilage and JSCs was not significantly different. The total amount of collagen from all the engineered groups was lower than that found in the native cartilage—generally about 50%. These results are in accordance with most of the published data obtained regarding tissue engineering cartilage with chondrocytes from young donors and chondrocytes from different anatomical sources[62, 80]. Therefore, it seems that the low production of collagen by chondrocytes on scaffolds is a fundamental problem of tissue engineering cartilage and not specifically dependent on donor age and species.

In addition to morphological and biochemical evidence that the engineered cartilages from both sources of cells were somewhat comparable, we sought to compare the biomechanical parameters of the new ECM generated by chondrocytes from juvenile animals and those from middle-aged humans. The reported equilibrium moduli of engineered cartilage generally are an order of magnitude lower than the native cartilage (juvenile swine cartilage: 457 ± 11 kPa; human native cartilage: 332 ± 12 kPa) from which the chondrocytes are collected. The results from confined compression testing of the nodules in this study show that the ECM from both groups is less stiff than native cartilage, but that the moduli in both groups are comparable. The weakness of the ECM could also be an artifact of the long-term subcutaneous implant model where the cartilage is not subjected to the normal biomechanical forces found in the joint.

The capacity of the cartilage to integrate with the existing cartilage is another characteristic important for the clinical application of engineered cartilage. Using a three-layered construct, we sought to determine whether the new cartilage would integrate with native cartilage by employing a tensile test. The Histological results demonstrated that the neotissue filled the intradisk space without any obvious gaps. Mechanical testing revealed that the UTS increased over time in the group using swine cells. Similar results were obtained in specimens using human chondrocytes at 12 and 18 weeks, but the UTS of human specimens at 24 weeks was much lower than the swine specimens.

It is also noteworthy that the temporal trends in compressive and tensile mechanical properties were quite different over the course of 24 weeks. Compressive properties declined from 18 to 24 weeks, when the equilibrium modulus was 2%–4% of native tissue and may be related to the drop in GAG production. In contrast, the tensile modulus of swine samples at 24 weeks (1.7 MPa) was 10%–20% that of native cartilage[81]. This is consistent with observations of continued collagen deposition at later time points and may suggest that long-term implantation in this system becomes more favorable for collagen network formation and less favorable for proteoglycan maintenance. Further, this is also consistent with other studies demonstrating that proteoglycan loss can enhance collagen network integrity, particularly in immature tissues[82]. Although the collagen content of specimens in the nodules from human and swine cells was not significantly different, the tensile properties of the three-layered human cell constructs show a weaker integration at 24 weeks. The collagen produced in the nodules may not be parallel to the collagen production in the three-layered constructs. Another explanation may be that the collagen produced between the cartilage disks in the three-layered constructs might not contribute to the integration strength in a linear fashion. Collectively, these data suggest that there are long-term (+ 18 weeks) remodeling processes that occur in tissue-engineered cartilage samples that affect the mechanical behavior of the tissue.

CONCLUSIONS

The results from this study demonstrated that chondrogenesis using articular chondrocytes from middle-aged people can be achieved in a predictable and reliable manner. Although the data are not identical to cartilage engineered with JSCs, there were many similarities noted in histological appearance, compressive moduli, and collagen production. Since most patients with articular cartilage lesions are not juveniles, but are quite often middle aged or older, it makes this head-to-head study comparing JSCs to human cells from middle-aged people to engineer cartilage more meaningful because it moves these cell–scaffold technologies closer to possible clinical application. Although the study design could have included groups from juvenile humans or older-aged animals, it was not logistically possible because obtaining cartilage from that human age group (5–15 years) is nearly impossible and old age, large animals are not commercially available. Further, the aim of this study was to determine whether cells from middle-aged, or even older, people have the capacity to generate cartilage ECM that is comparable to that observed by cells from juvenile animals, which has been the focus of most reported experimental cartilage engineering studies. Evaluating these parameters will help in developing strategies to apply tissue engineering approaches clinically.

ACKNOWLEDGMENTS

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Chapter 3

Conditions for seeding and promoting neo-auricular cartilage formation in a fibrous collagen scaffold

Zhao X, Bichara, DA, Zhou L, Kulig KM, Tseng A, Bowley CM, Vacanti JP, Pomerantseva I, Sundback CA, Randolph MA

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ABSTRACT

Carved autologous costal cartilage and porous polyethylene implants (Medpor®) are the most common approaches for total ear reconstruction, but these approaches may have inconsistent cosmetic outcomes, a high risk of extrusion, or other surgical complications. Engineering ear cartilage to emulate native auricular tissue is an appealing approach, but often the cell-seeded scaffolds are susceptible to shrinkage and architectural changes when placed *in vivo*. The aim of this study was to assess the most favorable conditions for *in vitro* pre-culture of juvenile animal cell-seeded type I collagen scaffolds prior to *in vivo* implantation. Sheep auricular chondrocytes (3-6 months) were seeded into this type I collagen scaffold. The cell-seeded constructs were cultured in either static or dynamic conditions for two days or two weeks and then implanted into nude mice for another six weeks. The harvested constructs were evaluated histologically, immunohistochemically, and biochemically. Robust neocartilage formation was found in these collagen scaffolds seeded with auricular chondrocytes, which was comparable to native cartilage morphologically, histologically, and biochemically. Culture under dynamic conditions prior to implantation improved the neocartilage formation histologically and biochemically. Dynamic culture of this sheep juvenile cell-seeded fibrous collagen material could permit predictable engineered auricular cartilage and a promising approach for external ear reconstruction.

INTRODUCTION

Reconstruction of the external ear due to congenital malformation or following traumatic injury is a challenging clinical problem in the field of plastic and reconstructive surgery. Since Gillies first carried out auricular reconstruction using autologous costal cartilage nearly a century ago, this approach has become the predominant surgical technique for partial and total reconstruction of the external ear[23]. Although reconstruction with carved autologous tissue often has favorable results, the cosmetic result is highly dependent on the sculpting abilities of the surgeon and implantation techniques. This technique has several major drawbacks including significant morbidity to the donor rib site[6], the potential for operative complications[24, 25], and these costal cartilage grafts are very stiff and inflexible. Costal cartilage grafts also are subject to absorption[26] and progressive calcification[27] over time.

An alternative strategy has been the implantation of alloplastic materials to simulate the human ear. Numerous alloplastic scaffold materials have been tried as framework substitutes for ear reconstruction, most of which have failed[83, 84]. Currently, porous polyethylene (Medpor®, Porex Surgical, Newnan, GA) is being used with clinical success to reconstruct and augment many areas of the craniofacial skeleton. Similar to carved costal cartilage grafts, however, porous polyethylene is very stiff and inflexible, and blunt force trauma to a Medpor® auricular implant can cause soft tissue breakdown leading to skin perforation, and possible infection resulting in implant removal[28]. Placing the Medpor® implant under a temporoparietal fascial flap[85] has eliminated some complications. However, reconstruction of the external auricle with an entirely synthetic rigid framework is not an optimal solution for all conditions. Engineered auricular cartilage may provide a solution for auricular reconstruction that behaves more like native ear cartilage[5]. For more than two decades, numerous groups have demonstrated that cartilage-like tissue can be generated *in vitro* and *in vivo* using chondrocytes or mesenchymal stem cells and a variety of biodegradable scaffold materials[62, 75, 86-90]. In 1997, Cao *et al*.[91] reported a human ear-shaped tissue engineered construct using bovine articular chondrocytes and a polyglycolic acid-polylactic acid scaffold. Several reports in the ensuing years have sought to improve engineered auricular tissue, but progress stalled because scaffolds could not maintain a satisfactory ear shape over longer time periods, even in immunodeficient mice models. Although numerous scaffolds support neocartilage formation in small animal models, no scaffold material has been developed that permits satisfactory cartilage generation under native inflammatory conditions in the subcutaneous environment of an immunocompetent large animal. Biodegradable scaffold materials including fibrin glue, alginate, and porous polymer scaffolds, when combined with chondrocytes have been useful in generating new cartilage matrix in small animals[92-95]. Scaffolds have been manufactured from a combination of materials such as polyglycolic acid (PGA), poly- L- lactic acid (PLLA), and polycaprolactone (PCL) to make human ear-shaped constructs[91, 96-102], and the mechanical and degradation requirements of synthetic materials can be modified and the polymers can be combined in various ratios to meet specific biological and biomechanical properties. For example, ear-shaped constructs containing polymers with slower degradation rates, such as PCL, were better preserved at the end of the studies because the shape of the auricle was maintained mainly by the persisting scaffold material[97-100]. However, the degradation products of the synthetic materials often cause chronic inflammation that can negatively affect neocartilage formation[103-106]. Furthermore, many of these scaffold materials degrade rapidly before adequate extracellular matrix has formed so that the construct is unable to withstand wound contraction forces imposed by the overlying skin, especially in large animal models. Natural biological materials are promising candidate scaffolds for cartilage engineering. Collagen, a major component of extracellular matrix (ECM), is natural protein that is abundant, biocompatible, and may eliminate the negative aspects of the degrading synthetic polymers on the neocartilage[107]. Although an immune response can be mounted to collagen-based products, advances in collagen purification and processing have rendered them relatively innocuous biologically[108]. Scaffolds made of collagen originating from diverse animal tissues are commercially available and have been actively used in research and clinical applications. For example, numerous biocompatible commercial products made from collagen have been used clinically for meniscus regeneration and osteochondral defect

repair[109, 110]. An initial report from our laboratory has shown that a wire-reinforced scaffold using collagen was capable of permitting cartilage formation, and the wire aided in maintaining the original dimensions of an ear-shaped construct in nude mice[111]. However, chondrocyte-seeded constructs without auxiliary support often shrink and change form and dimension upon immediate *in vivo* implantation[57, 62]. These dimensional changes could be disadvantageous for reconstructing an external auricle where size and shape are critical outcomes. One possible solution is to culture the cell-seeded constructs to initiate extracellular matrix formation prior to implantation with the goal of being able to predict the size and architecture of the final tissue engineered product. As part of a step-wise approach to engineer auricular cartilage, the optimal conditions for seeding cells and *in vitro* culture prior to *in vivo* implantation using a commercially available collagen scaffold currently in clinical use have not been determined. The source of animal cells chosen for this early preclinical study was sheep, which is a suitable model for obtaining autologous cells for implantation in future large animal studies. The aim of this study was to assess the most favorable conditions for *in vitro* pre-culture of cell-seeded type I collagen scaffolds prior to *in vivo* implantation.

MATERIALS AND METHODS

Scaffolds

The collagen scaffolds used in this study were manufactured from purified collagen extracts from bovine dermis, which is a source of primarily type I collagen (provided by Kensey Nash Corporation, Exton, PA, USA) with pore sizes ranging from 1.39 to 139.09 μm (16.34 ± 16.69 μm). Eighty discs measuring 5 mm diameter and 2 mm thick were punched from a sheet of fibrous collagen (Fig. 1). All scaffolds were sterilized with cold ethylene oxide gas prior to use.

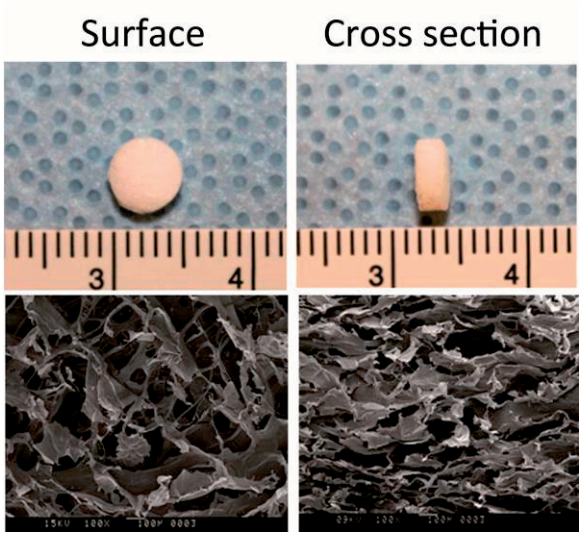

Figure 1. Gross appearance of the collagen scaffold showing the surface view (top left) and side view (top right). Scanning electron microscope images of the surface (bottom left) and a fracture surface cross section (bottom right) of the fibrous collagen scaffold.

Chondrocyte isolation and expansion

All actions were approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital. Ear cartilage was harvested from four, 3 to 6-month-old sheep donors. Under sterile conditions, the skin and perichondrium were carefully dissected and removed. One full-thickness piece of cartilage measuring 1 cm X 1 cm was collected from each sample and set aside for controls. The remainder of the cartilage was minced into 1 mm^3 fragments and digested in 0.1% type II collagenase (Worthington Biochemical, Lakewood, NJ, USA) for 16 — 18 h at 37 ^{0}C. After digestion,

the isolated chondrocytes were washed twice with PBS with antibiotics[62]. Cell number and viability were assessed using the trypan blue dye exclusion test. Cells were plated in roller bottles (Corning, Inc., Corning, NY, USA) at an initial density of 6 X 10^3 cells/cm^2, and cultured in Ham's F-12 medium with L-glutamine supplemented with 10% fetal bovine serum (SigmaeAldrich, St. Louis, MO), 50 mg/mL ascorbic acid, 100 U/mL penicillin, and 0.1 mM non-essential amino acids (chondrocyte medium) at 37 ^{0}C in a 5% CO2 until confluent. When the cells reached confluence, they were removed with 0.5% trypsin, washed twice in PBS, and counted. Five million sheep auricular chondrocytes were seeded onto each collagen scaffold disc in a 12-well culture plate. 2.5 million cells suspended in 0.1 mL PBS were pipetted onto the top of the scaffold and allowed to absorb into the porous collagen scaffold. The constructs were transferred into the incubator for 30 min, after which the constructs were flipped over and another 2.5 million cells in 0.1 mL PBS were seeded onto the other side. The scaffolds were flipped over at 20 min intervals for 3 h to allow the cells to infiltrate and attach to the scaffold prior to placing in culture.

Treatment groups

The constructs were cultured for either two days or two weeks in three different conditions at 37 ^{0}C in a humidified incubator with 5% CO_2 before implantation. In one group, constructs were cultured in static conditions. A second group of constructs was cultured in dynamic conditions on an orbital shaker (RotoMix mixer, Krackeler Scientific Inc., Albany, NY, USA) set at 40 rotations per minute (RPM)[112]. A third group of constructs was placed in a Rotational Oxygen-Permeable Bioreactor System (ROBS), which is a closed vessel with a gas permeable membrane set at 6 rotations per minute.

Construct implantation and harvest

After the seeded constructs were cultured for either two days or two weeks, two constructs from each group were collected for histological evaluation and the remainder (4-8 constructs) were implanted into subcutaneous pockets on the backs of nude mice (Cox-7 Laboratories, Massachusetts General Hospital, Boston, MA; four constructs per animal). Additionally, collagen scaffolds without cells (n = 4) were implanted into nude mice as unseeded controls. Under sterile conditions, the diameter and the thickness of each construct was measured with a digital caliper to the nearest tenth of a millimeter prior to implantation and at the time of harvest at 6 weeks. Similarly, the constructs were weighed under sterile conditions before implantation and at the time of explantation at 6 weeks. The constructs were randomLy selected and processed for morphological examination, histological analysis, or biochemical analysis. The constructs selected for biochemical analysis were stored at -80 ^{0}C until processed.

Histological analysis

Constructs from each treatment group at each time point, both *in vitro* and *in vivo*, were placed in 10% phosphate-buffered formalin for 24 h, embedded in paraffin, and sectioned. Cross-sections were prepared from a minimum of two areas from each construct and were stained with hematoxylin and eosin (H&E). Additional sections were stained with Safranin-O and Toluidine Blue to verify the production of sulfated glycosaminoglycans (GAG). Verhoeff's elastic stain kit (American MasterTech, Lodi, CA, USA) was selected to assess the presence of elastin in the constructs, as is standard practice reported in animal and human studies.

Immunohistochemical analysis

The constructs were evaluated for the presence of type II collagen indicative of the collagen in native sheep ear tissue and type I collagen, which could be produced by fibroblasts or dedifferentiated chondrocytes. Tissue sections were pretreated with 1 mg/mL pepsin in TrisHCl (pH 2.0) for 15 min at room temperature, followed by peroxidase block and serum block from M.O.M. kit (Vector Laboratories Inc., Burlingame, CA, USA). Sections were incubated with mouse anti-human collagen type I antibody (Accurate Chemical & Scientific Corp., Westbury, NY, USA) or mouse anti-human collagen type II antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) for 30 min. EnVision + System Peroxidase kit (Dako, Carpinteria, CA, USA) was used to identify the antigens; sections were counterstained with hematoxylin[111]. These antibodies were selected based on their

reactivity to sheep types I and II collagen and did not have any significant reactivity to the bovine collagen in the scaffold.

Biochemical analysis

Sample preparation

One 40-50 mg piece of native cartilage and engineered cartilage tissue was collected from each construct and weighed to obtain the wetweight before freezing at -80 ^{0}C. The samples were lyophilized for 24 h and weighed again to obtain the dry tissue weights. These samples were then subjected to biochemical analysis to determine the amounts of glycosaminoglycan (GAG) and hydroxyproline in the engineered cartilage tissue and native ear cartilage, reported as micrograms per unit weight (in milligrams) of dry tissue. Briefly, the samples for GAG and hydroxyproline analysis were digested with the addition of 1.0 mL of solution containing 100 mM sodium phosphate, 10 mM sodium ethylenediaminetetraacetic acid/disodium salt/dihydrate,10mMcysteine hydrochloride (SigmaeAldrich, St, Louis, MO, USA), 10 mM ethylenediaminetetraacetic acid (BDH, Poole, Dorset, England), and 125 µg/mL papain (Sigma-Aldrich). The samples were incubated in a 60 ^{0}C water bath for 24 h.

Glycosaminoglycan analysis

The GAG content of tissue was measured spectrophotometrically using dimethylmethylene blue dye from the Blyscan Glycosaminoglycan Assay kit (Biocolor, Ltd. Carrickfergus, UK) with chondroitin sulfate used as a standard. All samples and standards were analyzed in duplicate.

Hydroxyproline analysis

The hydroxyproline content of tissue digests was quantified with a previously described method[113]. Briefly, the papain digests were hydrolyzed with equal volumes of 6 N HCl at 115 ^{0}C for 16-24 h. Chloramine T hydrate (98%; SigmaeAldrich) and p-dimethylaminobenzaldehyde (Ehrlich's reagent; Sigma-Aldrich) were added to hydrolyzed samples, and absorbance was measured at 560 nm with a spectrophotometer immediately after addition of the dye. The amount of hydroxyproline in the samples was determined by using shark cartilage (Sigma-Aldrich) as a standard. All samples and standards were analyzed in duplicate. The amount of hydroxyproline was measured in micrograms per milliliter and reported as a percentage of dry tissue weight.

DNA analysis

DNA analysis followed the protocol using the Quant-iT™PicoGreendsDNA Kit (Molecular Probes, Invitrogen, Carlsbad, CA). A standard curve of DNA concentration was determined on the basis of absorbance at 260 nm (A260) in a cuvette with a 1 cm path length, and an A260 of 0.04 corresponded to 2 pg/mL dsDNA solution. The amount of DNA was calculated by comparing the absorbance of test samples to a standard DNA sample (µg/mL). The results were reported as percent of dry tissue weight.

Statistical analysis

Data were collected from quadruplicate samples and were expressed as the mean ± standard deviation (SD). Statistical analysis was performed with Student's t test and significance was determined at $p < 0.05$.

RESULTS

After six weeks *in vivo*, harvested constructs were surrounded by a thin, fibrous capsule that was easily removed. The constructs resembled cartilage in color and texture (Fig. 2) and were resistant to manual compression.

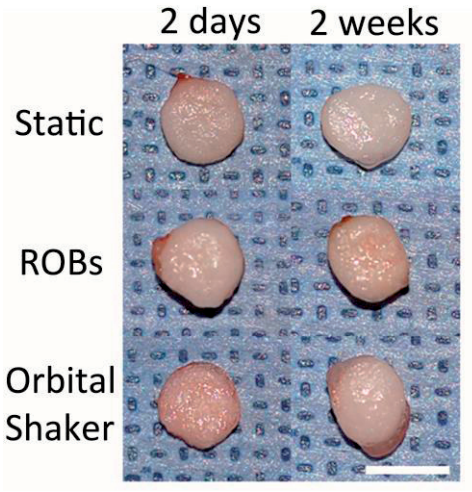

Figure 2. Constructs of harvested engineered cartilage made with sheep auricular chondrocytes and collagen scaffolds after six weeks subcutaneous implantation in nude mice. Constructs were pre-cultured under static conditions, ROBS, and on an orbital shaker for two days and two weeks (scale bar = 5 mm).

Changes in size and mass

The diameters of all constructs decreased after six weeks *in vivo*. Despite very small amounts of residual connective tissue, averaging measurements from three separate cross sections minimized any irregularities. The diameters of the constructs slightly decreased when cultured in static conditions for both two days and two weeks, but the diameter changes were not statistically significant. However, the diameters of the constructs cultured in dynamic conditions were substantially reduced, especially constructs cultured for two weeks in the ROBS (17 ± 3% decrease) and the orbital shaker (12 ± 2% decrease) constructs. The mass significantly decreased over six weeks implantation for all constructs cultured for two days; the mass was reduced by 45 ± 5% (static culture), 31 ± 4% (ROBS) and 43 ± 7% (orbital shaker) (Fig. 3A). There was no significant mass change for constructs cultured for two weeks under dynamic conditions before implantation; the mass decreased approximately 13 ± 15% (Fig. 3B).

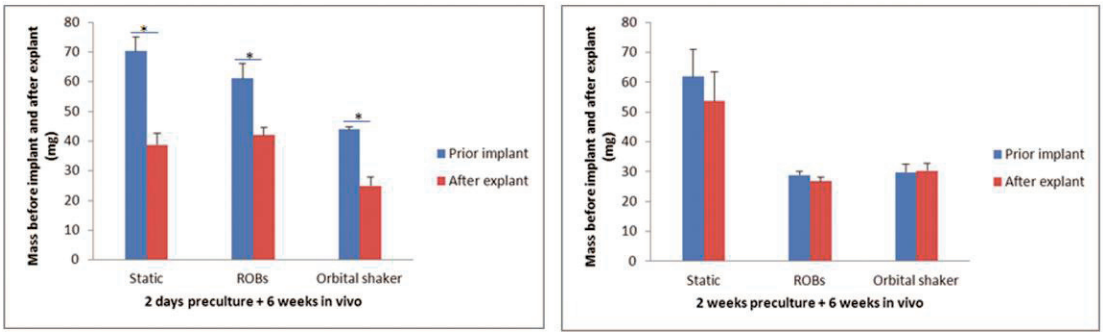

Figure 3. Changes in sample mass before and after implantation. There were significant differences in the mass of all samples pre-cultured for two days and harvested after 6 weeks of implantation in the three different conditions (*$p < 0.05$) (A). The mass of the samples pre-cultured in static conditions for two weeks and harvested after six weeks of implantation was reduced significantly (*$p < 0.05$). No significant difference in the

masses of samples could be detected when pre-cultured in ROBS and orbital shakers for two weeks before and after implantation (B).

Histological evaluation (*in vitro* and *in vivo*)

Histological evaluation of constructs cultured for two days showed chondrocytes dispersed throughout the scaffold matrix with little noticeable difference among the groups. The scaffolds cultured for two weeks showed increased numbers of cells in the interstices of the scaffolds and some matrix formation. Qualitatively, the constructs cultured in dynamic conditions for two weeks had appreciably more new matrix formation than those cultured in static conditions (*in vitro* histology not shown). For the constructs harvested from mice at 6 weeks, the nonseeded scaffold controls did not have any evidence of cartilage formation and only a few cells were noted in the interstices of the harvested scaffold (Fig. 4). New cartilaginous matrix was synthesized in all harvested scaffolds that were seeded with chondrocytes (Fig. 5). The morphology of the neocartilage appeared similar to native auricular cartilage, with cells located within typical chondrocyte lacunae and surrounded by cartilaginous matrix. However, the constructs cultured in dynamic conditions (ROBS and orbital shaker) showed more contiguous cartilage matrix than the constructs cultured in static conditions. Deposition of GAG was also evident by Safranin-O and Toluidine Blue staining. No significant morphological differences were observed in histological staining of the matrix and the cellular organization between the dynamically cultured constructs, although the matrices of constructs precultured for two weeks stained more intensely than matrices of constructs cultured for two days (Fig. 5). No vascular in-growth was observed in any of the constructs.

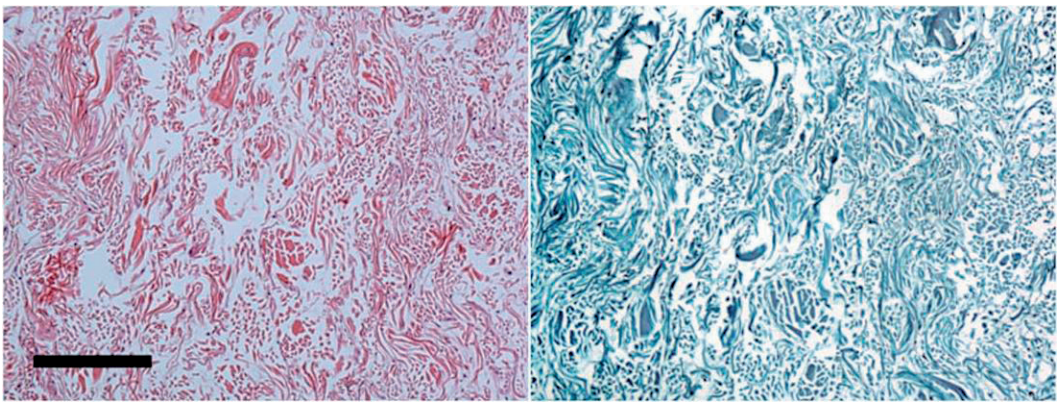

Figure 4. Histology from nonseeded bovine collagen scaffolds harvested from mice at six weeks. These images taken in the middle substance of the scaffold have few invading cells from the host animal and do not have any evidence of cartilage matrix formation (left stained with H&E; right stained with Safranin-O; Scale bar = 200 mm).

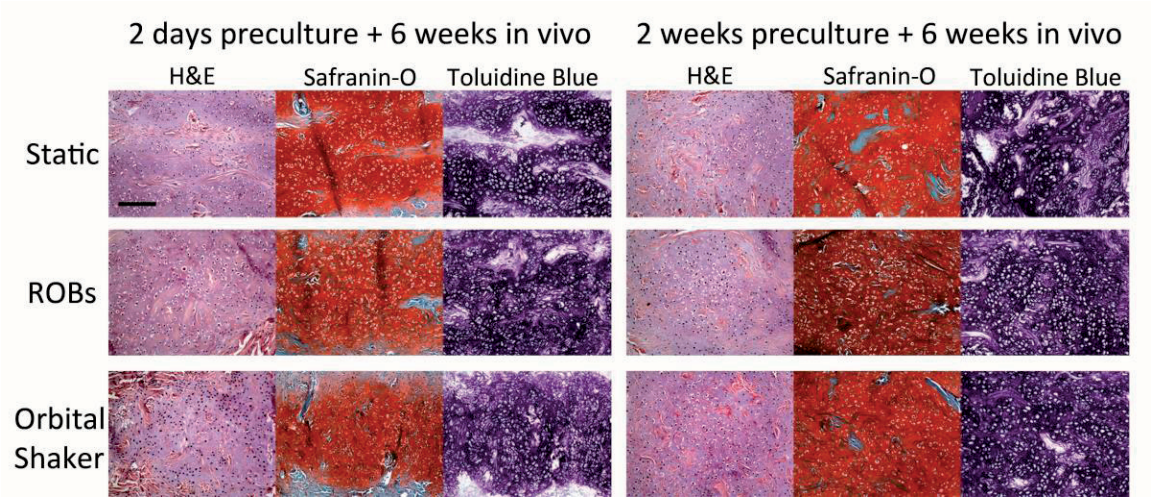

Figure 5. Histology of the cell-seeded constructs harvested at six weeks from nude mice. Constructs pre-cultured under static conditions, in ROBS, or on an orbital shaker for two days and two weeks. Each construct was stained with H&E to evaluate gross morphology of the neotissue and Safranin-O and Toluidine Blue to evaluate GAG in the new cartilage matrix. Unstained areas correspond to residual fibers of the collagen scaffold (Scale bar = 200 mm).

Immunohistochemical staining

Unseeded scaffolds were devoid of type II collagen and faintly stained for type I collagen (data not shown). The extracellular matrix in all the seeded constructs showed abundant type II collagen formation (Fig. 6) similar to the native ear cartilage tissue. There was little staining for type I collagen in any seeded construct.

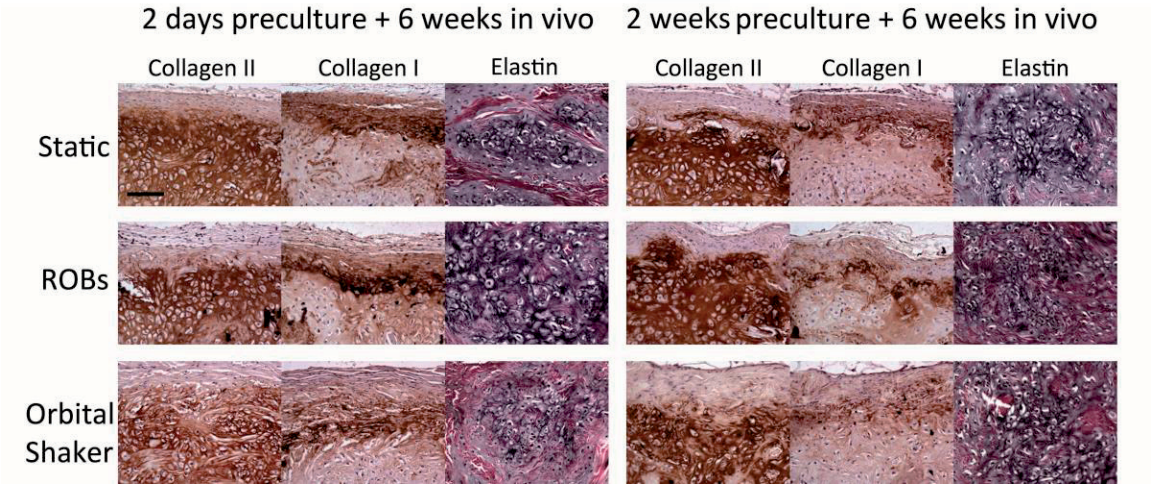

Figure 6. Immunohistochemical staining for type I and type II collagen and elastin staining of neocartilage constructs harvested at six weeks following pre-culture under static conditions, in ROBS, and on an orbital shaker. All new cartilage matrices stained intensely for type II collagen and there was little staining for type I collagen. Samples stained with Verhoeff's elastic stain depict elastin fibers (dark blue fibers) in the new tissue matrix (Scale bar = 100 mm).

Detection of elastin in harvested constructs

All constructs were examined for presence of elastic fibers using Verhoeff's elastic stain. Elastin was found in all constructs cultured under static and dynamic conditions (Fig. 6). Elastin staining appeared

more intense in constructs cultured for two weeks in contrast to constructs cultured for two days. Nonetheless, elastin in engineered cartilage was less dense than that observed in native sheep auricular cartilage.

Biochemical evaluation

Glycosaminoglycan analysis

There was a significant difference in the GAG content observed in constructs pre-cultured under the three different culture conditions (Fig. 7A). No significant difference was found in GAG contents between constructs pre-cultured in static conditions for two days and two weeks. However, the GAG content was significantly greater in constructs pre-cultured for two weeks in comparison for two days in both the ROBS and the orbital shaker. All GAG contents in engineered neocartilage were significantly higher than the GAG content in native sheep auricular cartilage.

Hydroxyproline analysis

The hydroxyproline contents of all engineered cartilage constructs were significantly lower than that of sheep native auricular cartilage tissue. No significant difference in the hydroxyproline content was observed for constructs pre-cultured under static or dynamic conditions for two days or two weeks pre-culture (Fig. 7B).

DNA analysis

All engineered cartilage constructs had significantly higher numbers of cells per milligram of tissue than sheep native auricular cartilage. No significant differences were observed in the DNA content of constructs pre-cultured under static or dynamic conditions for two days or two weeks (Fig. 7C).

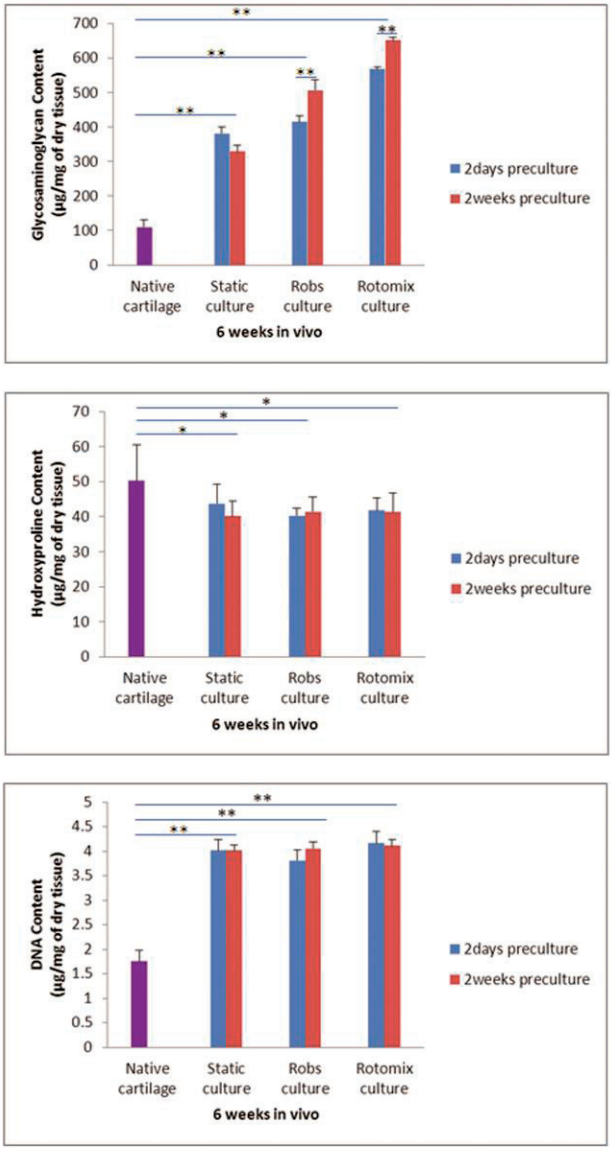

Figure 7. Biochemical analysis of engineered cartilage. (A) Amount of GAG in constructs harvested after six weeks *in vivo*. No significant differences were found in the GAG contents of samples pre-cultured in static conditions for two days and two weeks. Significant differences were observed in the GAG contents of samples pre-cultured in both ROBS and orbital shakers for two days and two weeks ($p < 0.05$). The GAG contents of all engineered samples were significantly higher than that of native cartilage. (B) Amount of hydroxyproline in the constructs harvested after 6 weeks *in vivo*. The hydroxyproline content in all engineered cartilage constructs was significantly lower than that of sheep native auricular cartilage tissue ($p < 0.05$). But no significant hydroxyproline content difference was found among all engineered cartilage constructs. (C) The amount of DNA in all engineered cartilage constructs was significantly higher than that of sheep native auricular cartilage tissue ($p < 0.05$). However, no significant differences in the DNA contents were found between all engineered constructs (*, $P < 0.05$; **, $P < 0.01$).

DISCUSSION

The overall goal for reconstructing the external auricle is to create an implant that will mimic the architecture and flexibility of the native ear and can be custom designed for the patient. Although stiff rib cartilage grafts and Medpor® implants can provide architectural similarity of the external auricle, a living tissue ear replacement would be an optimal choice for patients as it emulates the biocompatibility, durability, flexibility, and aesthetics of the native ear. To achieve this goal, several steps are necessary to generate suitable new tissue comparable to native ear cartilage. The initial step in this approach to grow ear cartilage was to assess the most favorable conditions for *in vitro* pre-culture of cell-seeded scaffolds prior to *in vivo* implantation to be able to predict the size of the final implant. Previous reports have shown that chondrocytes on various scaffolds decrease in size and mass after *in vivo* implantation[57, 62]. This presents a significant shortcoming for auricular reconstruction if an engineered construct shrinks after implantation. In an attempt to predict any decrease in size or mass, we postulated that *in vitro* culture may stabilize the constructs prior to *in vivo* implantation. The results showed that the constructs in culture for two days had lost some mass before implantation and continued to lose significant amounts of mass after being placed *in vivo* in all treatment groups making the end results fairly unpredictable. Samples that were cultured for two weeks decreased in mass during the *in vitro* culture period, and those in dynamic culture lost significant amounts of mass. However, the size and mass did not change significantly after *in vivo* implantation. Thus, the ending size of the constructs was more predictable after a longer *in vitro* culture period prior to *in vivo* implantation. These results suggest that a balance must be achieved between cartilage matrix formation *in vitro* and construct dimensional control upon implantation.

The histological results from this study demonstrated that this collagen scaffold provided a favorable environment for auricular chondrocytes to secrete new cartilage matrix. Constructs showed cartilage morphology with chondrocytes located within isolated lacunae surrounded by matrix. Constructs cultured under dynamic conditions (ROBS or orbital shaker) showed uniform matrix formation throughout the constructs, especially in samples precultured for the longer period of two weeks, compared with samples cultured under static conditions. Staining with Safranin-O and Toluidine Blue confirmed that the cells were producing GAG similar to chondrocytes in native ear cartilage. The engineered cartilage had intense type II collagen staining, which mimics the staining pattern observed in native auricular cartilage, with little type I collagen. Elastin, which plays a significant role in lending flexibility to native ear cartilage, was demonstrated in the new cartilage matrix of the constructs as early as six weeks *in vivo*. These results are similar to those reported by Xu *et al*. when using auricular cells in a fibrin gel scaffold[86]. Over these short *in vivo* periods, however, the staining of elastin was much less intense than native ear cartilage indicating that the new matrix was somewhat immature over the period of study.

Hypercellularity of new matrix is commonly observed in engineered cartilage[62, 75, 86, 114, 115]. The number of cells in the engineered cartilage estimated from the DNA content was more than double the cell number in native sheep ear cartilage, but no significant difference was found among samples in different groups. It is possible that mouse cells were capable of infiltrating the constructs and contributing to hypercellularity, but there is no evidence that invading mouse cells can form cartilage as confirmed by the cell-free scaffold controls placed in the mice. Similar to previous studies, the amount of GAG in neocartilage constructs was five to six-fold higher than in native sheep ear[62] (Xu *et al*., 2004). The amount of GAG in samples cultured in dynamic conditions was significantly higher than in constructs placed in static culture. It appears from these data that dynamic culture of chondrocyte-seeded constructs promotes superior extracellular matrix formation in comparison with static culture.

Most studies have reported hydroxyproline levels of engineered cartilage that average approximately 50% of native cartilage[60, 79]. The hydroxyproline content of the engineered cartilage in this study approached 80% of that found in native sheep auricular cartilage. Although the high hydroxyproline content of our engineered cartilage could be related to residual type I collagen fibers of the scaffold, the intense staining of type II collagen suggests scaffold turnover during neomatrix formation. More

studies are warranted to assess the remodeling and degradation of the collagen fibers in the collagen scaffold from type I to predominantly type II collagen as the cartilage matrix forms.

This is an early preclinical study performed in small animals and has several limitations. Sheep chondrocytes were chosen for this study because it is the model in which large animal tests will be performed. This model of sheep chondrocytes on bovine collagen scaffolds parallels the intended use of human chondrocytes on the bovine collagen when these studies reach clinical application. Since sheep have significant amounts of cartilage in their external auricle, we believe this is an appropriate model for the preclinical studies. Typical animal cell culture, as performed in this study, uses bovine serum and supplements that are not approved for human use. Such additives help to maximize the growth and differentiation of cells for early *in vivo* animal experimentation. As tissue engineering strategies progress to clinical use, the change to serum-free cell culture and approved additives can be made to facilitate translation into patients.

CONCLUSIONS

In summary, the results of this study suggest that this fibrous, type I collagen scaffold provides a favorable microenvironment for auricular chondrocytes to produce extracellular matrix. The engineered neocartilage is similar to native ear cartilage morphologically, histologically, and biochemically. Pre-culture of the cell-seeded scaffolds for two weeks in dynamic conditions (ROBS and orbital shaker) improves neocartilage formation histologically and biochemically, and may permit predictive outcomes on the physical architecture of the neocartilage. Engineered auricular cartilage using this collagen-based scaffold could be a promising alternative for external ear reconstruction. Additional studies in small and large animals will be performed to determine the feasibility of ear reconstruction using this material as a scaffold for auricular regeneration.

ACKNOWLEDGMENTS

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Chapter 4

Chondrogenesis by bone marrow-derived mesenchymal stem cells grown in chondrocyte-conditioned medium for auricular reconstruction

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Submitted for journal publication.

ABSTRACT

Bone marrow-derived mesenchymal stem cells (BMSCs) can be obtained by minimally invasive means and would be a favorable source for cell-based cartilage regeneration. However, controlling the differentiation of the BMSCs towards the desired chondrogenic pathway has been a challenge hampering their application. The addition of exogenous growth factors may aid in chondrogenic differentiation, but is insufficient and associated with the risk of indefinite proliferation, endochondral bone formation, and tumorigenesis. The major aim of this study was to determine if conditioned medium collected from cultured auricular chondrocytes could promote chondrogenic differentiation of BMSCs. Auricular chondrocytes were isolated and grown in BMSC standard culture medium (SM) that was collected and used as chondrocyte-conditioned medium (CCM). BMSCs were expanded in either CCM or SM for 3 passages. Cells were then seeded onto fibrous collagen scaffolds and pre-cultured for two weeks with or without transforming growth factor-beta 3 (TGF-β3). After pre-culture, constructs were implanted subcutaneously in nude mice for 6 and 12 weeks and evaluated with real-time PCR, histology, immunohistochemistry, and biochemistry. Real-time PCR results showed up regulation of COL2A1 in the constructs cultured in CCM compared to those in SM. After 12 weeks *in vivo*, abundant neo-cartilage formation was observed in the implants that had been cultured in CCM, with or without TGF-β3. In contrast, very little cartilage matrix formation was observed within the SM groups, regardless of the presence of TGF-β3. Osteogenesis was only observed in the SM group with TGF-β3. In conclusion, CCM even had a stronger influence on chondrogenesis than the supplementation of the standard culture medium with TGF-β3, without signs of endochondral ossification. Efficient chondrogenic differentiation of BMSCs could provide a promising alternative cell population for auricular regeneration.

INTRODUCTION

Total ear reconstruction still remains one of the most challenging issues in plastic and reconstructive surgery. Microtia-atresia, anotia, trauma, infections, or neoplasms of the intricately shaped external auricular cartilaginous structures are conditions that may require multiple surgical procedures for reconstruction [2]. The prevalence and characteristics of microtia vary in different populations and ranges widely from 0.83 to 17.4 per 10,000 [116]. Currently, the most recommended treatment for auricular reconstruction is a multi-staged technique using carved autologous costal cartilage [117]. Due to the challenges for shaping harvested autogenous rib cartilage, the cosmetic results of the auricular reconstruction can be highly variable and often unsatisfactory—particularly in unskilled hands [118]. Although carved rib cartilage can provide favorable three-dimensional architecture, costal cartilage grafts are stiff, inflexible, and can undergo resorption over time. The use of a Medpor® auricular implant made of porous polyethylene has been another alternative for ear reconstruction [85]. Like the costal cartilage, however, Medpor® has limited flexibility, and extrusion has been reported in both patients and animal models [28, 119].

A tissue-engineering approach could be employed to generate structurally durable ear replacements that better match both the functional properties and appearance of native ears [2]. In combination with biofabrication technologies, stable constructs with intricate three-dimensional shapes could be generated [120]. However, one challenge to successfully achieving such a tissue engineered auricle is the identification of a suitable cell source providing sufficient numbers of cells to generate an ear. In comparison to chondrocytes (both autogenic and allogenic), which have been used as the primary source for auricular cartilage engineering, stem cells have a number of advantages and may be used as an alternative cell source. Stem cells are a self-renewing cell population with an ability to undergo multilineage differentiation [33]. These cells are multipotent and able to differentiate into osteoblasts, adipocytes, myoblasts and, more importantly, chondrocytes that could be applied in engineering a human auricle [34].

One of the greatest challenges for using mesenchymal stem cells (MSCs) to generate cartilage is directing the cells down the desired chondrogenic differentiation pathway [35]. Many different kinds of growth factors have been studied to achieve chondrogenic differentiation with varying results [36, 121]. Among those, transforming growth factor-beta 3 (TGF-β3) has been shown to be one of the most efficient growth factors for inducing chondrogenic differentiation of stem cells [122, 123]. However, MSCs may exhibit a hypertrophic phenotype under chondrogenic induction resulting in calcification after ectopic transplantation [38, 39]. While exogenous recombinant growth factors have been shown to be useful in laboratory studies, most have not been approved by the U.S. Food and Drug Administration (FDA) for clinical use [37].

Several studies have shown the possibility of improving chondrogenesis through the co-culture of the stem cells with chondrocytes [39-46]. All of these studies, however, focused on the co-culture of stem cells with articular chondrocytes rather than with auricular chondrocytes. Additionally, the stem cells and chondrocytes were often mixed together in the co-culture environment, which can confound the contribution to matrix formation between the two different cell populations. Several of these studies have claimed that increased cartilage formation in these co-cultures is mainly due to a trophic role of the MSCs in stimulating chondrocyte proliferation and matrix deposition by the chondrocytes rather than the MSCs actively undergoing chondrogenic differentiation [49, 50].

Chondrocytes are capable of producing numerous soluble growth factors and cytokines that are released into the medium [48]. If so, the medium collected from chondrocyte cultures, referred to as chondrocyte-conditioned medium (CCM), could be effective in inducing chondrogenesis of bone marrow-derived MSCs (BMSC). A few studies have been reported using CCM to encourage MSCs to produce articular cartilage matrix molecules [124, 125]. However, none have applied similar strategies for auricular cartilage generation. Hence, the major aim of this study was to determine if CCM collected from cultured auricular chondrocytes could promote chondrogenic differentiation of BMSCs towards auricular cartilage matrix formation. For comparison, we used the standard protocol of using exogenous recombinant TGF-β3 to differentiate the MSCs.

MATERIALS AND METHODS

Harvest and isolation of sheep auricular chondrocytes and bone marrow mesenchymal stem cells (BMSCs)

All animal studies were approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital. Auricular cartilage was harvested from three- to six-month-old Polypay sheep. The sheep ears were shaved, washed, and prepped with povidone iodine solution. The skin and perichondrium were carefully dissected and removed under sterile conditions. A full-thickness piece of cartilage measuring 1 cm x 1 cm was collected from each sample and set aside for controls. The remainder of the cartilage was minced into 1 mm3 fragments and washed three times in Dulbecco's phosphate buffered saline (PBS) with antibiotics (Sigma, St. Louis, MO, USA). The minced cartilage fragments were suspended in 0.1% type II collagenase (Worthington Biochemical, Lakewood, NJ, USA) and digested for 16-18 hours at 37°C. After digestion, particulate debris was removed by passing the digest solution through 100 m nylon cell filters. The isolated chondrocytes were rinsed and washed twice with PBS containing antibiotics. Cell viability was determined using trypan blue dye and a hemacytometer. Only cell isolations with >90% viability were used for the subsequent studies. Cells were plated in roller bottles (850 cm2) (Corning, Tewksbury, MA, USA) at an initial density of 6×103 cells/cm2 and cultured in BMSC standard medium (SM) composed of high glucose DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 50 mg/ml ascorbic acid, 100 U/ml penicillin, and 0.1 mM non-essential amino acids at 37°C in 5% CO2. Medium was changed three times per week. Collection of the chondrocyte-conditioned medium started when the cells exceeded 50% confluence. The media was removed using a sterile pipette and filtered through a 0.2μm filter to remove any cells and re-sterilize the media. The CCM was stored at 4oC and used within one week for the subsequent studies.

Bone marrow was collected from the sheep by aspiration from the iliac crest under sterile conditions using an 8 gauge bone marrow biopsy needle (World Wide Medical Technologies, Oxford, CT, USA). The mononuclear cell fraction from each bone marrow sample was obtained by Ficoll-Paque Plus (GE Healthcare, Otelfingen, Switzerland) separation of low-density cells from red blood cells and granulocytes. After cell counting, cells were resuspended in MesenPRO RSTM Medium (Life Technologies, Grand Island, NY, USA), plated in 150 cm2 vented culture flasks at a density of 6×103 cells/cm2, and incubated in a humidified atmosphere at 37oC with 5% CO2. After one week, the flasks were washed with PBS and the nonadherent cells were removed. The adherent cells were cultured until they reached subconfluency at which time they were harvested by trypsinization for use according to treatments outlined below.

Growing BMSCs using chondrocyte conditioned medium

Following the initial culture phase to isolate the BMSCs, the cells were plated into 850 cm^2 roller bottles (Corning) at an initial density of 6×10^3 cells/cm^2 for further proliferation [126]. Half of the BMSCs were cultured in CCM collected from sheep auricular chondrocytes, and the other half of the cells were cultured in SM as control. All of the cells were incubated at 37°C in 5% CO_2 and the cells were grown through two passages under these conditions. Medium was changed three times per week. The cells were harvested from the roller bottles with 0.05% trypsin-EDTA (Invitrogen, Grand Island, NY, USA) when they reached 90% confluence and washed twice with PBS for the next studies placing the cells on scaffold material.

Materials—collagen scaffold

The fibrous collagen scaffolds used in this study were manufactured from purified collagen extracts from bovine dermis, which is primarily type I collagen (provided by DSM Biomedical, Exton, PA, USA). We previously demonstrated the feasibility of ear cartilage regeneration using auricular chondrocytes on this collagen scaffold [111]. Eighty discs measuring 5 mm in diameter and approximately 2 mm in thickness were punched from a sheet of fibrous collagen using a dermal biopsy punch. All scaffolds were sterilized with cold ethylene oxide gas prior to use.

Construct assembly and preculture

The sheep BMSCs that had been proliferated in CCM or SM were seeded onto collagen scaffold discs (3x106 cells/disc) and cultured in the presence or absence of TGF-β3. Using a pipette, 1.5 million cells suspended in 50 µl PBS were seeded onto the top of the scaffold, and the constructs were transferred into the incubator for 30 minutes. The constructs were removed from the incubator, flipped over, and another 1.5 million of cells suspended in 50 µl PBS were seeded onto the bottom side. Then the samples were flipped at 20 minute intervals for three hours to allow the cells to infiltrate and attach to the scaffold.

The BMSC-seeded scaffolds were cultured in serum-free media, with or without TGF-β3. Serum-free medium was prepared with high glucose DMEM (Sigma) containing 2 mM L-glutamine, 100 nM dexamethasone (Sigma), 50 µg/ml ascorbic acid phosphate (Sigma), 1 mM sodium pyruvate (Invitrogen), 40 µg/ml proline (Sigma), 1% ITS+ (BD Biosciences, Franklin Lakes, NJ, USA) and with or without TGF-β3 (10 ng/ml, R&D Systems, Minneapolis, MN, USA). Four groups of constructs were studied and the group names describe the conditions of BMSC proliferation and the conditions in which the BMSC-seeded discs were cultured.

Group 1; CCM + TGF-β3

Group 2; SM + TGF-β3

Group 3; CCM –TGF-β3

Group 4; SM – TGF-β3

All constructs were cultured for two weeks *in vitro* prior to implantation in nude mice. Three samples from each treatment group were harvested on days 1 and 14 for histological processing and real-time PCR analysis.

Real-time PCR analysis.

On days 1 and 14 of the pre-culture period, real-time PCR was performed to determine if the BMSCs were producing cartilage-specific mRNA as previously described [127]. Total RNA was isolated from BMSCs using the TRIzol method (Invitrogen). Total mRNA (0.5 µg) was reverse-transcribed using SuperScript reverse transcriptase with random hexamer. The real-time PCR was performed using SYBR Green PCR Mastermix and the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). cDNA samples were analyzed for the gene of interest and using β-actin as the reference gene. Sequences of primers were 5’ TGGCACCACACCTTCTACAATGAGC 3’ and 5’ GCACAGCTTCTCCTTAATGTCACGC 3’ for β-actin; 5’ GAAACCATCAATGGTGGCTTCC 3’ and 5’ GATAACAGTCTTGCCCCACTT 3’ for type II collagen (COL2A1). The level of expression of each target gene was then calculated as $-2\Delta\Delta Ct$.

***In vivo* implantation**

Following *in vitro* culture for two weeks, the samples were implanted into subcutaneous pockets on the dorsum of nude mice. Specimens were harvested at 6 and 12 weeks and examined grossly for the appearance of cartilage-like tissue. The specimens were randomly assigned for histological processing or biochemical analysis. The specimens selected for biochemical analysis were stored at -80oC until processing.

Histological analyses

The randomly selected specimens from each group at each time point, both *in vitro* and *in vivo*, were placed in 10% phosphate-buffered formalin for 24 hours, embedded in paraffin, and sectioned. Cross-sections were prepared from a minimum of two areas from each specimen and were stained with hematoxylin and eosin (H&E). Other sections were stained with Safranin O to verify the production of sulfated glycosaminoglycan (GAG) and toluidine blue to visualize cartilage matrix. Verhoeff’s elastin staining kit (American MasterTech, Lodi, CA, USA) was used to assess the presence of elastin. All the specimens were stained with Alizarin Red solution to evaluate whether calcium deposition occurred in the new tissue matrix. The sections were evaluated in a blinded manner by three independent reviewers to determine the presence or absence of neo-cartilage formation.

Immunohistochemical staining

Immunohistochemistry was performed on tissue sections from each group for collagen type I and II. The tissue sections were pretreated with 1mg/mL pepsin in Tris HCl (pH 2.0) for 15 minutes at room temperature, followed by peroxidase block and serum block from M.O.M. kit (Vector Laboratories Inc., Burlingame, CA, USA). Sections were incubated with mouse anti-human collagen type I antibody (Accurate Chemical & Scientific Corp., Westbury, NY, USA) or mouse anti-human collagen type II antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) for 30 minutes. EnVision+ System Peroxidase kit (Dako, Carpinteria, CA, USA) was used to identify the antigens; sections were counterstained with hematoxylin [111].

Biochemical analyses

After 6 weeks and 12 weeks *in vivo*, 4-5 samples from each group were analyzed biochemically as previously described [127]. Briefly, lyophilized specimens were digested with papain solution (125 μg/mL papain type III, 100 mM phosphate, 10 mM l-cysteine, and 10 mM EDTA, pH 6.3) at 60°C for 16 hours. After digestion, cell debris and insoluble materials were removed by centrifugation at 6,000 g for 5 minutes. Portions of the digest were analyzed for DNA using Quant-iT^{TM} PicoGreen dsDNA Kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA), and sulfated GAG by Blyscan Glycosaminoglycan Assay kit (Biocolor, Ltd. Carrickfergus, UK)

DNA analysis

A standard curve of DNA concentration was determined on the basis of absorbance at 260 nm (A260) in a cuvette with a 1 cm path length. An A260 of 0.04 corresponds to 2 pg/mL dsDNA solution based on a standard curve calibration. The amount of DNA was calculated through the comparison between the absorbance of test specimens and DNA standard samples provided in the kit. The results were reported as μg DNA/mg of dry tissue weight.

Glycosaminoglycan analysis

Following the protocol of Blyscan Glycosaminoglycan Assay Kit, the microplate reader was set to 656nm. The absorbance against water for the reagent blanks, standards and test samples was measured. Glycosaminoglycan (GAG) concentrations were obtained from the Standard Curve. All samples and standards were analyzed in duplicate, and the duplicates should be close to ±5% of their mean value. The results were reported as μg GAG/mg of dry tissue weight. In addition, GAG content was normalized against total DNA content and reported as μg GAG/μg DNA.

Statistical analysis

Data were collected from quadruplicate samples and were expressed as the mean+/-standard deviation (SD). Statistical analysis was performed with Student's t test and ANOVA. Significance was determined at $p< 0.05$ and $p< 0.01$.

RESULTS

In vitro study

Real-time PCR

The relative expression COL2A1 mRNA was low in all groups on day 2 of culture. Expression was significantly higher after 14 days of culture, especially in the CCM groups (Fig. 1). The COL2A1 expression in the CCM+TGF-β3 group was upregulated 1488-fold; expression in the SM+TGF-β3 group was upregulated 9-fold; expression in the CCM-TGF-β3 group was upregulated 232-fold; and expression in the SM-TGF-β3 group only increased 2-fold.

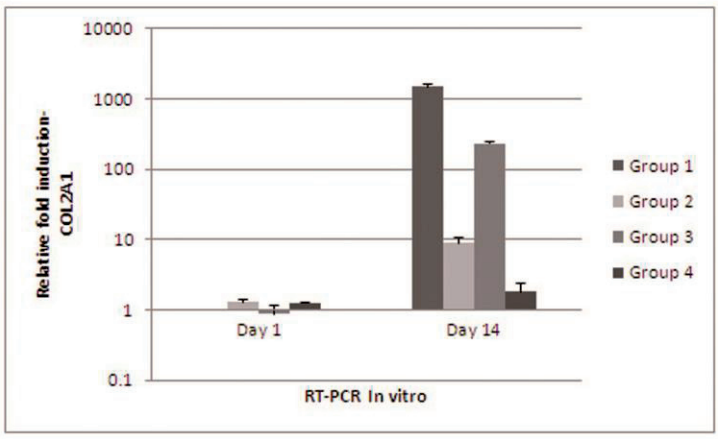

Figure 1. RT-PCR analysis. The messenger RNA expression of COL2A1 of all of the four groups at days 1 and 14 *in vitro*. Group 1: CCM+TGF-β3; Group 2: SM+TGF-β3, Group 3: CCM-TGF-β3, and Group 4: SM-TGF-β3. At day 1, only minimal COL2A1 was measurable. By day 14 the expression of COL2A1in groups treated with CCM was significantly higher than groups treated with SM. (**p<0.01)

Histological analyses

Histological evaluation of samples cultured for two days *in vitro* showed cells dispersed throughout the scaffold matrix with little noticeable difference among the groups (Fig. 2). The samples cultured for two weeks *in vitro* all showed increased numbers of cells in the scaffolds and some extracellular matrix (ECM) formation. Qualitatively, there was more ECM formation in the CCM groups that appeared denser around the margins of the disc.

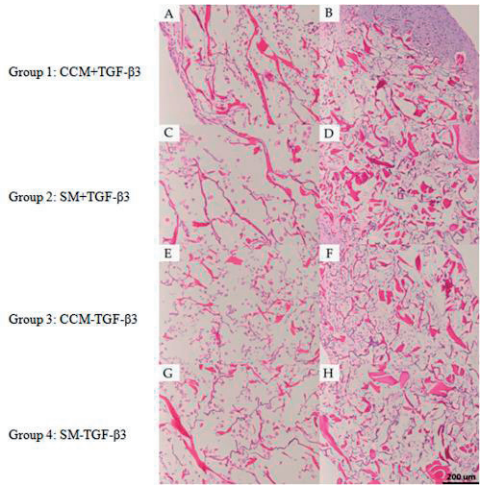

Figure 2. Photomicrographs of the samples from all of four groups (CCM+TGFβ-3, A & B; SM+TGFβ-3, C&D; CCM -TGFβ-3, E&F; SM - TGFβ-3, G&H) after 2 days and 14 days *in vitro* preculture (2 days, A, C, E, G; 14 days, B, D, F, H). By day 14 there were appreciably more cells within the scaffolds in all groups (B,D,F,H). In Group 1 treated with CCM+TGFβ-3 at day 14, new matrix could be noted in the scaffold (B). (Hematoxylin and eosin staining; scale bar is 200 µm)

In vivo **study**

Histological analyses

In constructs harvested from mice at 6 weeks, new cartilaginous matrix had begun to form in the samples of the CCM groups (data not shown). The chondrocytes in the newly formed tissue demonstrated similar morphological characteristics as those in native auricular cartilage, with cells

located within typical chondrocyte lacunae and surrounded by cartilaginous matrix. By 12 weeks *in vivo*, the histological results revealed significantly more contiguous cartilage matrix deposition than was observed at 6 weeks in the CCM groups. Some ECM was visualized in the SM groups at both time points, evidenced by scant staining with Safranin O or toluidine blue. No evidence of vascular in-growth was noted in any of the *in vivo* specimens.

All specimens were examined for presence of elastic fibers by staining histological sections with Verhoeff's elastin staining. Elastin, detected as a dark mesh-like pattern of staining surrounding chondrocytes, was observed in the CCM groups after 12 weeks *in vivo,* regardless of the presence or absence of TGF-β3 treatment. Elastin was not observed in groups grown in standard medium, even in the presence of TGF-β3.

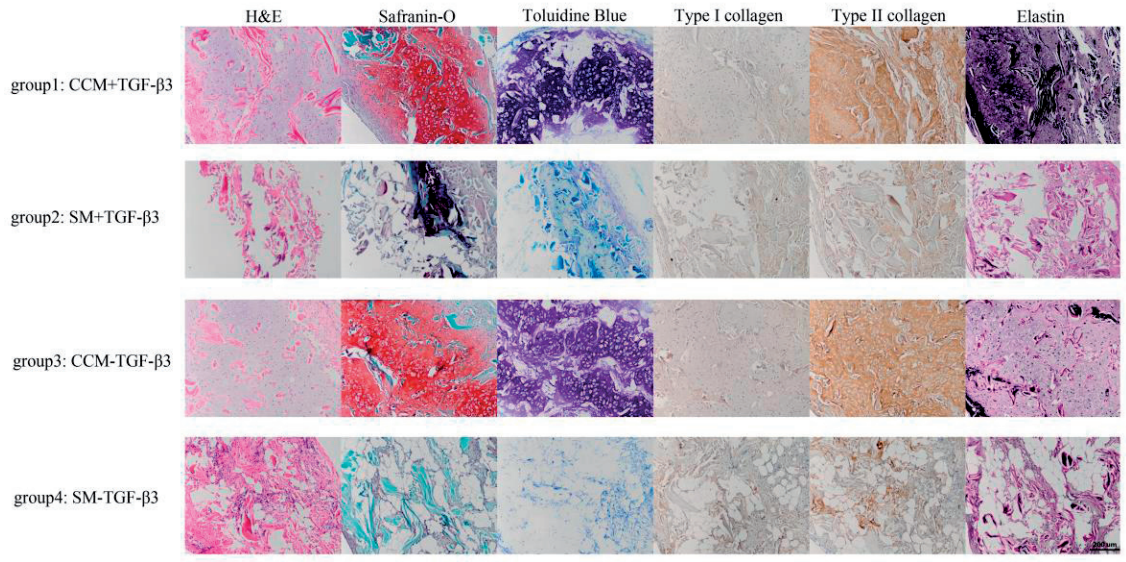

Figure 3. Photomicrographs of the samples harvested at 12 weeks from nude mice. New cartilage matrix can be noted in the specimens treated with CCM, with or without TGFβ-3, stained with Safranin O and toluidine blue. Similarly, these two groups also stained more intensely for collagen type II and elastin, observed as small dark fibers in the matrix. (H&E, Safranin O, Toluidine Blue, type I&II collagen, and elastin staining; scale bar is 200 μm)

Immunohistochemical staining

Immunohistochemical analysis demonstrated that unseeded scaffolds were devoid of type II collagen and only faint staining of the scaffold material was noted for type I collagen (data not shown). The ECM in samples treated with CCM showed abundant type II collagen staining and very little type I collagen staining at 12 weeks (Fig. 3). Very little type II collagen was found in groups treated with SM at both time points analyzed (Fig. 3).

Alizarin Red staining

All specimens were examined for presence of calcium deposits by staining histological sections with Alizarin Red S staining. No calcium deposits were found in the CCM groups and the SM group that was not exposed to TGF-β3, both at 6 and 12 weeks, in line with histological findings of sheep native auricular cartilage. However, some calcium deposits were noticed in the SM group pre-cultured in the presence of TGF-β3 (Fig. 4).

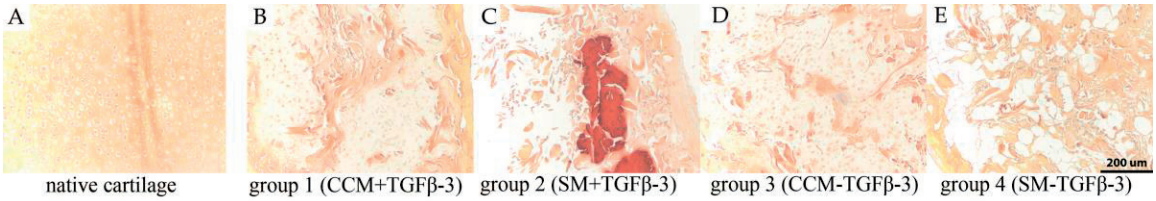

Figure 4. Alizarin Red staining of the samples harvested at 12 weeks from nude mice (B-E). Only Group 3 treated with SM plus TGFβ-3 showed positive staining. Native sheep auricular cartilage was stained for comparison (A). (scale bar is 200 μm)

Biochemical analyses

DNA analysis

At 12 weeks, the amount of DNA for each group was 8.2±0.7 μg/mg dry tissue in the CCM+TGF-β3 group; 4.5±0.6 μg/mg dry tissue in the SM+TGF-β3 group; 7.5±0.7 μg/mg dry tissue in the CCM-TGF-β3 group; and 8.7±0.5 μg/mg dry tissue in the SM-TGF-β3 group. All groups were higher than the DNA content of sheep native auricular cartilage, which was 3.9±0.4 μg/mg dry tissue suggesting the newly formed cartilage tissue was hypercellular.

Glycosaminoglycan analysis

After 12 weeks of implantation, the amount of GAG for the groups was as follows: 49±0.5 μg/mg dry tissue in the CCM+TGF-β3; 14±0.3 μg/mg dry tissue in the SM+TGF-β3; 29±0.6 μg/mg dry tissue in the CCM-TGF-β3; and 22±0.3 μg/mg dry tissue in the SM-TGF-β3. Only the group treated with CCM plus TGF-β3 was higher than the glycosaminoglycan content of sheep native auricular cartilage, which was 41±0.5 μg/mg dry tissue.

GAG content was also normalized against total DNA content. The normalized amount in the CCM+TGF-β3 group was 5.9±0.5 μg GAG/μg DNA; in the SM+TGF-β3 group it was 3.1±0.2 μg GAG/μg DNA; in the CCM-TGF-β3 group it was 3.9±0.4 μg GAG/μg DNA; and in the SM-TGF-β3group it was 2.5±0.5 μg GAG/μg DNA. By this measure, all groups were less than sheep native auricular cartilage, which was 10.5±0.3 μg GAG/μg DNA (Fig. 5).

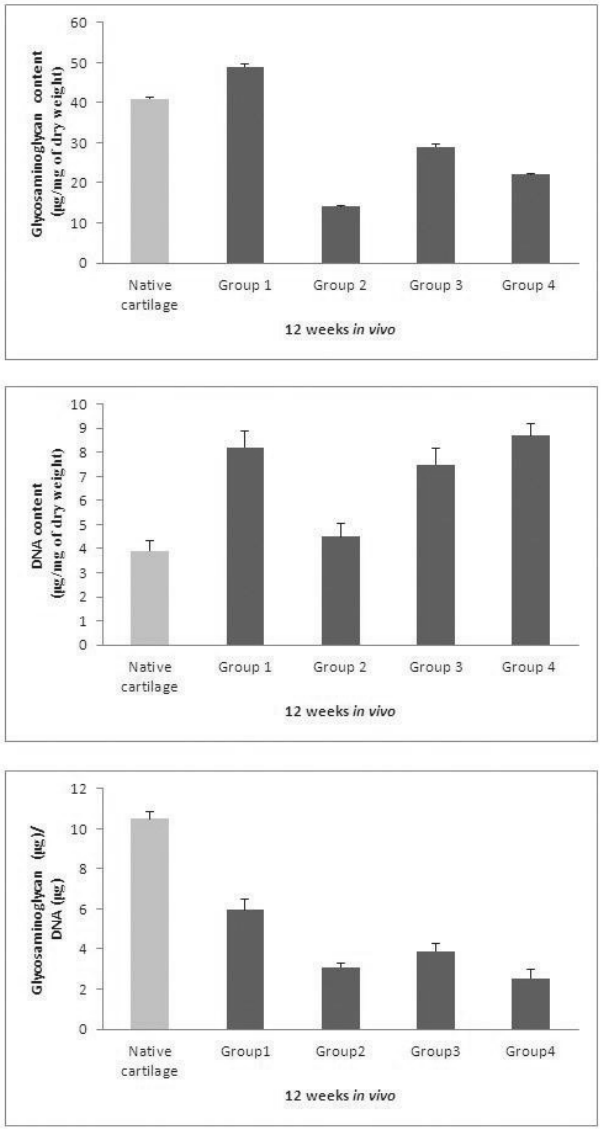

Figure 5. Biochemical analyses of the samples harvested at 12 weeks from nude mice compared to sheep native auricular cartilage. The overall GAG content in Group 1 treated with CCM plus TGFβ-3 was higher than native sheep, whereas all other groups were lower than native (top). The amount of DNA in groups 1, 3, and 4 were all higher than native cartilage suggesting they were hypercellular (middle). When measured as GAG/DNA, however, all groups were lower than native cartilage, yet the groups treated with CCM were higher than groups in SM (bottom). (**$p<0.01$; *$p<0.05$)

DISCUSSION

Tissue engineering has become a promising technique to achieve regeneration of auricular cartilage defects. A number of studies have demonstrated proof of principle for generating auricular cartilage using primary auricular chondrocytes and various scaffold materials [86, 128, 129]. Some studies have extended these strategies by forming the auricular cartilage into the shape of the external ear [91, 111, 120, 130]. Although these are promising strategies, obtaining sufficient numbers of primary auricular chondrocytes to generate a human-sized ear may not be easily achieved. Alternative sources of chondrogenic cells could permit wider application of tissue engineering approaches for ear reconstruction.

MSCs isolated from bone marrow are multipotent cells that are able to differentiate into a variety of cell types including chondrocytes. MSCs could potentially be applied to reconstruct auricular cartilage, but the biggest challenge is driving the differentiation towards the desired tissue type. In the recent years, a wide range of growth factors have been used to stimulate chondrogenesis of MSCs, predominantly in *in vitro* settings. These growth factors include transforming growth factor β (TGFβ) family, mainly TGF-β1, -2, and -3, bone morphogenetic proteins (BMPs)-2, -4, -6, -7 and -9, fibroblast growth factor 2 (FGF-2) and insulin-like growth factor 1 (IGF-1)[36, 121]. The regulating role of TGF-β3 in cellular differentiation and cartilage formation was first highlighted in 1986 by similarities found during its comparison to cartilage inducing factor-A [36]. Since then, an increasing number of studies have showed that TGF-β3 plays an important role in chondrogenesis [131, 132]. Further studies showed that TGF-β3 helped in producing more type II collagen in human BMSC and human adipose-derived MSC cultures than TGF-β1 and TGF-β2 [133, 134]. Mara *et al*. also concluded that TGF-β3 used in micromass culture is the best growth factor for promoting the proliferation and differentiation of mesenchymal stem cells from umbilical cord blood during chondrogenesis, and it was better than IGF-1 [135]. All these growth factors are effective for differentiating stem cells at some level, however, most have not been approved by FDA for clinical use due to concerns of biologic, economic, and strategic issues [37].

Although the addition of exogenous recombinant growth factors are effective for differentiating MSCs, we focused this study on finding a means to promote stem cell-based auricular cartilage generation without having to add growth factors. Co-culture of different cell types is based on the idea that the multi-signal events *in vivo* cannot be perfectly mimicked by adding a limited variety of growth factors to a monoculture, of which the optimal cocktail remains largely elusive. This problem has been circumvented by several investigators by the introduction of another cell source in the culture to expose the cells to a wider variety of naturally produced stimuli [40-46, 122]. The stimuli are believed to be the secreted soluble factors from the additional cells.

Several reported studies have revealed that chondrocytes in culture are capable of producing and releasing numerous growth factors and cytokines, including cytokine-like protein 1 (Cytl1), bone morphogenetic protein-2 (BMP-2), parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), TGF-β1 [127], insulin-like growth factors 1 and 2 (IGF-1, IGF-2) [46], fibroblast growth factor 4 (FGF-4), and insulin-like growth factor-binding proteins-4, 6 (IGFBP-4, 6) [136], and others. Hypothetically, we could capitalize on all of those endogenous factors secreted by the chondrocytes into the culture medium by collecting the medium used to grow chondrocytes (chondrocyte-conditioned medium) and using it as a growth factor cocktail to differentiate MSCs. These naturally produced soluble factors could be a preferred method of stimulation, as it omits the issues regarding the use of nonautologous recombinant factors in clinical settings [47]. This strategy also avoids the direct contact between the different cell sources. Thus, the primary objective of this study was to evaluate the chondrogenic differentiation of BMSCs cultured in chondrocyte-conditioned medium using the growth factors produced normally by auricular chondrocytes. The ultimate goal was to use these cells that had been primed *in vitro* with CCM to generate auricular cartilage *in vivo.*

Collagen type II alpha 1, also known as COL2A1, is a human gene that provides instructions for the production of the pro-alpha1(II) chain of type II collagen. This gene encodes the alpha-1 chain of type II collagen, a fibrillar collagen found in native cartilage tissue [49]. Type II collagen is a significant

component of the ECM of auricular cartilage, and it defines the biomechanical properties of the tissue. Type II collagen mRNA is recognized as a marker for chondrogenesis in chondroprogenitor cells. Through the real-time PCR analysis, we found that the expression of COL2A1 mRNA in BMSCs cultured in CCM was significantly higher than BMSCs cultured in SM after 14 days of *in vitro* pre-culture. Interestingly, the CCM group without TGF-β3 had a much higher expression of COL2A1 mRNA than the SM group with TGF-β3, demonstrating that the chondrocyte-conditioned medium outperformed the addition of exogenous TGF-β3 in promoting chondrogenesis under our study conditions. Although TGF-β3 has been shown to be effective for chondrodifferentiation on MSC pellet cultures, the dispersed placement of the MSCs on the collagen scaffold may have decreased its effect in this study.

The histological results in samples comprised of BMSCs cultured in CCM showed increased cellular proliferation and matrix deposition after 2 weeks of *in vitro* pre-culture over those grown in SM. After 12 weeks of *in vivo* implantation, the neocartilage matrix was relatively uniform in the groups treated with conditioned medium compared to those treated with the standard medium. Histology of specimens of the CCM groups revealed a cartilage-like morphology of the tissue with chondrocytes located within isolated lacunae and intense Safranin O staining confirming abundant GAG deposition. The immunohistochemical results showed the engineered cartilage in the CCM specimens stained intensely for type II collagen whereas only limited staining was observed for type I collagen. However, the samples exposed to standard medium contained very little type II collagen. Another important component in auricular cartilage is elastin, which provides flexibility to ear cartilage. Through the elastin staining, we observed that elastin fibers were present in both CCM groups at 12 weeks. Nonetheless, the elastin in the tissue-engineered cartilage was still less dense than that observed in native auricular cartilage [128]. By contrast, elastin was undetectable in the SM samples. Considering the importance of elastin in native ear cartilage, the results above are very meaningful for the potential to engineer auricular cartilage. Based on the histological analyses, the engineered cartilage constructed with BMSCs cultured in CCM had a much better performance than cells cultured in SM, which indicates CCM is a more potent stimulator of chondrogenesis than SM under the conditions of this study.

GAG is one of the most important components in ECM of native auricular cartilage tissue. Our study showed that the amount of GAG per DNA of the CCM groups was significantly higher than that in the SM groups, which means the engineered cartilage tissue constructed with BMSCs proliferated in CCM generated more cartilage matrix than those grown in SM. It was not surprising that the CCM+TGF-β3 group had the highest amount of GAG, which is not surprising, because it was simulated by both CCM and the supplementation of TGF-β3. The results of the GAG/DNA in all groups were still lower than native cartilage, probably because the chondrogenic differentiation pathway is neither thoroughly understood nor complete. Since the scaffold used in this experiment was made of type I collagen, it was not possible to use a total collagen assay like hydroxyproline content as a reliable measure of collagen production. However, we were able to detect type II collagen distribution by immunohistochemical staining.

During *in vitro* differentiation, mesenchymal stem cells can undergo hypertrophy and eventual calcification, an unfavorable result for cartilage generation [39, 127, 137]. Alizarin Red S staining showed that there were no obvious calcium deposits in any of the samples of the CCM groups at all time points, indicating that there was no calcification, at least throughout the 2-week *in vitro* culture and subsequent 12-week *in vivo* implantation. It remains an important issue, however, in view of the long-term maintenance of the auricle *in vivo*. Interestingly, the only group showing any calcium deposits was group 2 (SM+TGF-β3). This suggests that the application of TGF-β3 alone may induce BMSCs towards osteogenesis, which could be a major disadvantage for auricular cartilage regeneration when using purified growth factors. Several studies have reported that TGF-β isoforms (TGF-β1 and TGF-β3) induced hypertrophy during chondrogenesis of chondroprogenitor cells [138-140]. Interestingly, Fischer *et al*. found that human articular chondrocytes (HAC)-derived soluble factors are potent means of improving chondrogenesis and suppressing the hypertrophic development of MSCs [43]. Bigdeli *et al*. also demonstrated that co-culture of human embryonic stem cells and

human articular chondrocytes further resulted in a significantly decreased osteogenic potential [40]. Our results are consistent with those previous reports.

Co-culture strategies have shown great potential for inducing chondrogenesis and a few applications have been translated to the clinic [141]. However, very few studies have focused on the specific mechanisms of interaction between MSCs and chondrocytes and the synergistic action of MSCs and chondrocytes on chondrogenesis. While several studies have demonstrated successful chondrodifferentiation of MSCs *in vitro* where conditions can be carefully controlled, our study expanded this potential by placing the constructs *in vivo* and successfully generating cartilage matrix. We did not assess the growth factor profile of the CCM and that will be the topic of future studies. As such, more studies are needed to understand the mechanisms involved between CCM treatment and chondrogenesis. More testing in animal models, especially in large immunocompetent animals, will be performed in the future.

CONCLUSIONS

In conclusion, chondrocyte-conditioned medium (CCM) was effective in inducing chondrogenic differentiation in BMSCs. Specifically, CCM had a stronger influence on chondrogenesis than supplementation of the standard culture medium with TGF-β3 without inducing calcification. MSCs could provide a source for a large number of cells for the generation of adult size human ear if strategies for efficient chondrogenic differentiation could be determined without the need for exogenous recombinant growth factors. The utilization of the chondrocytes' own growth factor production to stimulate differentiation of MSCs is an appealing approach. Since few studies have reported on auricular cartilage generation using conditioned medium or co-culture strategies, these results could provide a foundation for new strategies in auricular reconstruction.

ACKNOWLEDGEMENTS

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Chapter 5

Enhancing the stiffness of collagen hydrogels for delivery of encapsulated chondrocytes to articular lesions for cartilage regeneration

Omobono MA, Zhao X, Furlong MA, Kwon CH, Gill TJ, Randolph MA, Redmond RW

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ABSTRACT

This study investigated a dual crosslinking paradigm, consisting of (a) photocrosslinking with Rose Bengal (RB) and green light followed by (b) chemical crosslinking with 1-ethyl-3-(three-dimensionalimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to enhance gel stiffness while retaining chondrocyte viability. In group 1, 50 μL collagen constructs of 2% (w/v) type I collagen containing 10 μM RB were allowed to gel spontaneously at 37°C. In Group 2, the spontaneous gels were exposed to green light (532 nm) alone. In Group 3, the photochemically crosslinked gels were subsequently treated with a 1 hour exposure to 33 mM EDC/6 mM NHS. Samples (n=18) were subjected to 0.08% (w/v) collagenase digestion, and the storage modulus of samples was measured using a rheometer. The viability of encapsulated chondrocytes was measured by a live/dead assay. Chondrocytes were ≥95% viable in all constructs at 10 days *in vitro*. Resistance to collagenase digestion increased in the order spontaneous gels (2h) < photochemical gels (3-4 h) < dual crosslinked gels (>24h). The storage modulus of dual-crosslinked constructs was increased 5-fold over that of both photocrosslinked and spontaneous gels. Photochemical ± chemical crosslinking of collagen I hydrogel did not reduce encapsulated chondrocyte viability, while the dual-crosslinked collagen gels demonstrated an increase in stiffness and resistance to collagenase digestion. These crosslinked collagen hydrogels could be a useful tool for the practical delivery of encapsulated chondrocytes to articular defects.

INTRODUCTION

Articular cartilage has little innate ability to heal and presents significant clinical challenge for natural regeneration. It is populated exclusively by chondrocytes[142], but is avascular and lacks the capacity for complete, spontaneous regeneration in response to any focal injury to the contiguous extra-cellular matrix (ECM). Any procedure aimed at restoring the articular surface is required to (a) create natural hyaline cartilage to fill the defect and (b) allow integration of the neocartilage with existing healthy cartilage.

Among many potential natural and synthetic matrices in tissue engineering, collagen has proven to be useful in hydrogel[143, 144], membrane[145, 146], and porous scaffold[147, 148] forms. Collagen is naturally occurring in human tissue, including articular cartilage[149]. Collagen molecules self-assemble into a hydrogel matrix at physiological pH and temperature via hydrogen bonding but these spontaneous hydrogels are weak in mechanical integrity and are rapidly digested by enzymatic attack. In an attempt to overcome these drawbacks, we exposed collagen gels containing photoreactive dyes to visible light to induce crosslinking of adjacent collagen molecules. These photocrosslinked gels were protected from contraction due to cellular interaction with the matrix[150] and chondrocytes encapsulated in photocrosslinked collagen hydrogels formed hyaline-like cartilage[75]. However, these gels are relatively soft and difficult to handle, and the aim of this work was to modify the crosslinking process to provide a stiffer collagen hydrogel that would be more user-friendly in ultimate clinical use.

Any increase in gel stiffness must be achieved without affecting the viability of encapsulated chondrocytes or their ability to remodel the existing type I collagen scaffold and generate neocartilage. The feasibility of implanting a collagen hydrogel in an articular surface defect hinges on the mechanical integrity of the hydrogel during implantation and early repair. During this early period chondrocytes must have a scaffold that supports viability and permits new cartilage formation. Photocrosslinked gels may lack the stiffness necessary to withstand extended mechanical loading and an enhancement in construct stiffness could be beneficial.

Chemical crosslinking using 1-ethyl-3-(three-dimensionalimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) is non-toxic and useful for crosslinking collagen in tissues[151-155]. EDC, in the presence of NHS, activates carboxyl groups of aspartic and glutamic acid residues of collagen to react with nucleophilles, such as primary amines (lysine and hydroxylysine) and hydroxyl groups, to create zero-length crosslinks[156]. Treating collagen hydrogels with EDC/NHS is a potential method for increasing crosslinking, and the combination of photo- and chemical crosslinking could yield a hydrogel with improved stiffness[157].

In this study, we have used dual-crosslinking (photochemical + chemical) of type I collagen hydrogels to test whether the stiffness of collagen gels can be substantially increased to make them useful for articular cartilage defect repair. Effects on mechanical properties of the gel, encapsulated chondrocyte viability *in vitro*, and resistance to collagenase digestion were evaluated to determine the potential of this new crosslinking paradigm for articular cartilage regeneration.

MATERIALS AND METHODS

Materials

Rat tail type I collagen (11.4 mg/mL) was obtained from BD Biosciences, Bedford, MA. Ham F12, 10% fetal bovine serum. 1% antibiotic/antimycotic liquid and 1% MEM non-essential amino acids were all from Gibco (Carlsbad, CA). Sodium hydroxide (NaOH), Rose Bengal (RB), 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide, N-hydroxysuccinimide and N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) were purchased from Sigma-Aldrich (Natick, MA). Type II collagenase at 245 U/mg was purchased from Worthington Biochemical (Lakewood, NJ).

Preparation of collagen hydrogels

Rat tail type I collagen was mixed in an Eppendorf tube with chondrocyte media [Ham F12 , 10% fetal bovine serum (Gibco), 1% antibiotic/antimycotic liquid , 1% MEM non-essential amino acids], 50 mM stock NaOH , and 90 μM Rose Bengal diluted in chondrocyte media. The collagen solutions were adjusted to pH 7.2 ± 0.2 using 50 mM NaOH. The final concentrations were 10μM Rose Bengal and 2.54 mg collagen / mL.

Fifty μL of the hydrogel mixture was aliquoted into cylindrical molds of 4.7 mm diameter Spontaneous gelation of the collagen solution was induced by incubation at 37°C for 1 hour. Photochemically crosslinked gels were prepared by exposure to green light from a continuous wave KTP laser (LRS-0532-PFH-000500-05, Laserglow Technologies, Canada, 800mW, 532nm) at 20J/cm^2 fluence from three different angles to assure equal illumination throughout the specimen. A group of photochemically crosslinked gels also underwent subsequent chemical crosslinking following ejection from the mold by submerging in a solution of 33 mM EDC, 6 mM NHS, and 50 mM HEPES buffer for 1 hour. Another group of gels was prepared solely by exposure to EDC/NHS for 60 minutes without prior photochemical crosslinking.

In order to compare concentration and time of exposure to EDC/NHS solution, all collagen gels were prepared in a similar fashion through green laser exposure. Samples were then subjected to increasing dilutions and increasing times of exposure (n=3 per group) according to Table 1.

Table 1. Group designations for EDC/NHS concentration and time of exposure study

Dilution [EDC]/[NHS] (m*M*)	15-Min Exposure	30-Min Exposure	45-Min Exposure	60-Min Exposure
33/6	1-15	1-30	1-45	1-60
16.5/3	2-15	2-30	2-45	2-60
6.6/1.2	5-15	5-30	5-45	50-60
3.3/0.6	10-15	10-30	10-45	10-60

Collagenase digestion assay

Collagen hydrogel constructs were submerged in 10 mL of 0.08% w/v type II collagenase to determine the degree of protection from enzymatic degradation provided by crosslinking treatments in 15 mL conical tubes (BD Biosciences). Tubes were placed on a lab rocker at room temperature and checked every 15 minutes to determine the length of time for complete dissolution of the construct. Gels that were still intact after 24 hours were recorded as “undigested”. Three individual trials were conducted with n=6 per trial for a total of n=18 samples per group.

Mechanical testing

The viscoelastic storage modulus, G’, of each collagen construct was measured using a rheometer (TA Instruments AR-G2, 8mm Rough Steel Smart-Swap plate, part #511080.906). Samples were subjected to a frequency sweep from 1-10 radians/second with constant 2% strain rate and an 800 μm gap under continuous, room-temperature oscillation. Shear elastic moduli were calculated from the stress measured, using the equation;

$$G' = (\sigma 0 / \epsilon 0) \cos(\delta)$$

where $\sigma 0$ is the stress applied, $\epsilon 0$ is the strain measured and δ is the measured lag between phases. Three trials were conducted at n=6 per group per trial for a total of n=18 samples per group.

Chondrocyte viability

Knee joints were obtained from euthanized, four-month old Yorkshire swine and dissected under sterile conditions to expose the femoral condyles and the posterior face of the patella. The cartilage was dissected from the knee and digested in 0.1% w/v type II collagenase solution overnight at 37°C. Digested cartilage solutions were passed through a 100 μm cell strainer and centrifuged at 250g for 10 minutes. The cell pellet was collected and washed twice with fresh chondrocyte media. Cells were counted on a hemocytometer and tested for initial viability using the trypan blue exclusion assay. Chondrocytes were plated in monolayer at a density of 2 x 106 cells/150 cm^2 and cultured at 37°C and 5% CO2 until 80% confluent. Cultured plates were then exposed to 0.05% trypsin-EDTA (Invitrogen) and cells were collected in chondrocyte media, and washed twice with fresh media.

Cells were then suspended in collagen hydrogel mixture at 1.0 x 10^7 cells / mL hydrogel (n=4). Gels were molded, incubated, and crosslinked as described above. After crosslinking treatment, gels were submerged in chondrocyte media for *in vitro* culture. After 10 days the gels were submerged in a solution of Live/Dead Viability Assay (Invitrogen, Grand Island, NY), containing 1.6 μM calcein AM and 200 nM ethidium homodimer-1, for 1 hour. The stained constructs were embedded in O.C.T. Compound (Tissue Tek), frozen at -20°C for 1 hour, and sliced into 10 μm sections using a cryostat (Leica CM3050). These slices were then imaged using a Nikon Eclipse (TE2000U) fluorescence microscope using FITC (480ex/535em) and TRITC (535ex/610em) filters, and processed with NIH ImageJ software.

Statistics

Results of collagenase and mechanical testing data are reported as mean ± standard deviation. Significance was calculated using 1-way ANOVA analysis with Bonferroni's Multiple Comparison Test post-test with $p < 0.05$ considered significant. Percentage chondrocyte viability was calculated by counting dead (red) and live (green) cells from the fluorescent photographs. Samples were photographed in triplicate, capturing one central image and two boundary images per sample. Both live and dead cells were counted manually from which the viable fraction of cells was calculated. Manual counting was performed by 5 independent evaluators and results are presented as the mean of the five independent viability percentage for each gel ± standard deviation.

RESULTS

Resistance to collagenase digestion

Full degradation was defined as complete dissolution of the hydrogel by collagenase. Collagen hydrogels photocrosslinked with Rose Bengal and green laser exposure (t_{deg} = 3.5 ± 0.5 hours) exhibited > 2-fold increase ($p < 0.0001$) in time of degradation from the spontaneous gels (1.3 ± 0.3 hours). Dual-crosslinked constructs digested in collagenase solution exhibited no signs of degradation, even after 24 hours. Gels that were crosslinked with EDC/NHS alone for 60 minutes were soft but very resistant to degradation; after 24 hours there were no signs of degradation (Fig. 1). Exposure of constructs to different concentrations of EDC/NHS for different times showed varying degrees of resistance to digestion (Fig. 3). Within a treatment dilution group, t_{deg} increased with increasing time of exposure to EDC/NHS. Between treatment dilution groups, t_{deg} increased with increasing concentration of EDC/NHS.

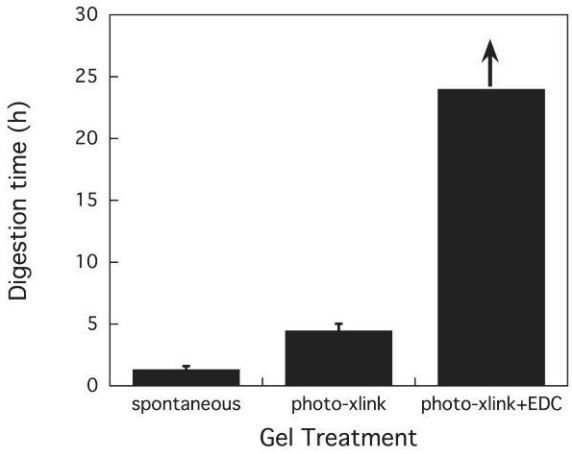

Figure 1. Times of degradation of 2% w/v collagen gels of various crosslinking treatments when exposed to 0.08% w/v collagenase enzyme at room temperature (n = 6 per group). Photocrosslinked gels lasted significantly longer (3.46 ± 0.13 h) than spontaneous gels (1.32 ± 0.06 h) ($p<0.0001$). Gels exposed to dual crosslinking were completely undigested after 24 hours.

Mechanical testing

Values for storage modulus in spontaneous control gels (25.8 ± 1.5 Pa) and storage modulus in photocrosslinked gels (21.4 ± 1.8 Pa) showed no statistically significant difference ($p = 0.0712$). Storage modulus measurements for dual-crosslinked gels were 5-fold higher (117.6 ± 6.9 Pa) than both photocrosslinked and spontaneous gels ($p < 0.0001$). (Fig. 2). Gels exposed to dilute concentrations of EDC/NHS (1:10, 1:5) did not show any significant difference in storage modulus from uncrosslinked and photocrosslinked groups (Fig. 4). Groups exposed to EDC/NHS diluted 1:2 for at least 15 minutes showed a trend of increasing storage modulus with increasing exposure time.

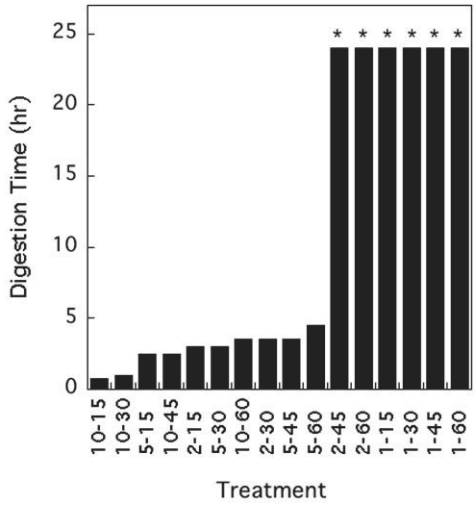

Figure 2. 0.08% w/v collagenase digestion of 2% w/v collagen gels treated with different dilutions and exposure times of EDC/NHS. Refer to Table I for group designations. Gels labeled with an asterisk (*) did not digest after 24 hours, when the study was capped.

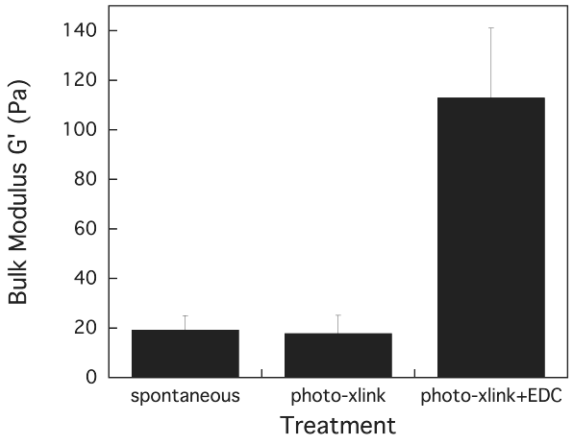

Figure 3. Storage modulus G' as measured by a rheometer of 2% w/ v collagen gels of various crosslinking treatments (n = 6 per group). There was no significant difference between spontaneous gels (25.8 ± 1.5 Pa) and photocrosslinked gels (21.4 ± 1.8 Pa) (p = 0.58). There was a significant difference, up to a 5-fold increase in storage modulus when collagen gels were treated with dual crosslinking (117.6 ± 6.9 Pa) (p<0.0001).

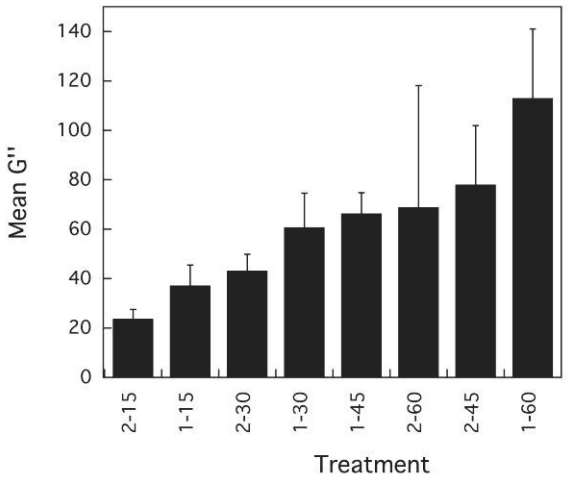

Figure 4. Storage modulus (G') by rheometry of 2% w/v collagen gels treated with different dilutions and exposure times of EDC/NHS. Refer to Table I for group designations. There is no statistical significance between gels of groups exposed to lower concentrations of EDC/NHS. The first significant difference is noticed at group 2–30, with varying degrees of stiffness modulation between group 2–30 and maximum crosslinking treatment, group 1–60.

Chondrocyte viability

Chondrocytes exhibited 96.1 ± 2.3% viability in hydrogel implants after one week of *in vitro* culture (Fig. 5). Cells residing in the peripheral regions of the constructs tended to have a lower viability than those in the central regions of the gels. Overall, the viability far exceeded our target viability of 90% for encapsulated chondrocytes to produce healthy ECM.

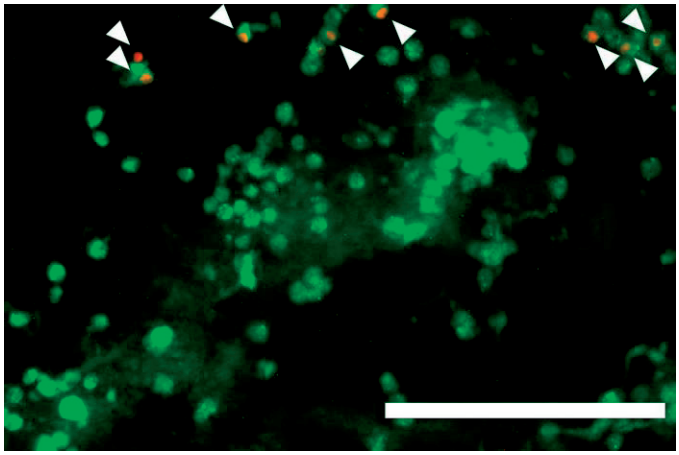

Figure 5. Live/Dead photographs of dual crosslinked construct after 10 days of *in vitro* culture. Pictures are taken at a height of 750µm ± 50µm from the base of each construct in a 10µm cryotome slice. Dead cells are denoted by white arrows and scale bar is 300 µm.

DISCUSSION

Articular cartilage regeneration is a challenging clinical problem. The newest tissue-engineering-based therapy, autologous chondrocyte implantation (ACI), involves biopsy of healthy hyaline cartilage from a non-weight bearing area of the knee to harvest autologous chondrocytes for expansion *in vitro* followed by injection of cultured cells to the cartilage defect. The cells are typically suspended in saline and a periosteal flap is sutured to the cartilage surface. Not only does this repair require multiple surgeries to complete, but there is little data supporting the benefits of ACI versus microfracture[158] or OATS[159]. A common problem during this procedure is the leaking of cell-saline suspension from under the periosteal flap out of the target site due to compressive pressure despite sealing the surgical site with suture and fibrin glue.

Finding a suitable matrix to support cellular activity and ECM generation is a common problem in tissue engineering[76, 152, 160, 161]. Native articular cartilage consists of different types of collagen fibrils, but is mostly type II collagen by dry weight8. Type I collagen is the main structural component in many native tissues in the body, therefore it is an attractive option as a non-toxic, biomimetic matrix to support natural ECM regeneration by chondrocytes. Collagen hydrogels can be formed at 37 °C but these "spontaneous" gels are very soft and are unsuitable for implantation in focal articular cartilage defects.

Photocrosslinking of synthetic polymers typically is well-established and typically uses ultraviolet (UV) illumination of photoinitiator compounds that react with synthetic monomers to cause chain reaction polymerization via radical reactions. However, for the purpose of cell encapsulation the UV illumination and the materials used would not be appropriate due to inherent cytotoxicity[162]. For this reason we have investigated visible light as the energy source for crosslinking along with non-toxic initiators. Under these milder conditions the initiating event can be electron transfer between excited initiator and monomer and/or energy transfer from photoinitiator to dissolved oxygen to form singlet oxygen and its subsequent reactions, (e.g. oxidation of histidine and reactions of photooxidized histidine with other amino-containing residues[163] to form a crosslink[164].

We have previously shown that photochemical crosslinking of a collagen hydrogel provides a scaffold that supports encapsulated chondrocytes and stimulates cartilage-like ECM production[75, 150]. Photochemical crosslinking certainly stabilizes the matrix and makes it more resistant to enzymatic degradation but provides little in the way of additional mechanical stiffness that would enhance practical handling of the gel and remove the need for a covering material to be affixed over the cartilage defect to prevent loss of unstable gel. Thus, we sought an improved crosslinking mechanism that enhances initial stiffness of these collagen-based hydrogels.

An obvious possibility would be to increase the fluence (photons/cm^2) incident on the gel. However, we have already shown in cell-free collagen gels that there is a plateau in the fluence dependence of crosslinking, suggesting a saturation of all sites that can be photochemically crosslinked, and further exposure does not equate to increased crosslinking. As an aside, this approach would also be complicated by side reactions of the photoinitiator that can generate reactive species that contribute to cell toxicity, as shown in previous studies. Thus, other methods are required and led to the investigation of chemical crosslinking of collagen-based matrices with a combination of EDC and NHS. This method has been shown to increase mechanical stiffness of materials[151-153, 156, 160, 165], including organized tissues, without cytotoxic effects.

We investigated this method alone and in combination with photocrosslinking as a method for increasing collagen hydrogel stiffness. Experiments using EDC/NHS crosslinking alone on collagen gels were disappointing. Constructs exposed to this treatment were only loosely organized and collapsed under their own weight after being ejected from the mold. These gels could not be subjected to storage modulus evaluation. Despite a lack of structural integrity, these gels proved highly resistant to collagenase digestion, showing that crosslinking did, in fact, occur. Since the clinical application requires implantation of snug-fitting gels with a defined geometry, crosslinking with EDC/NHS alone is not an option. However, when photocrosslinking was practiced prior to EDC/NHS treatment a stiffer gel construct was obtained that did retain its shape when extruded from the mold. Thus, a dual crosslinking paradigm has potential to provide a practical implant for focal defect repair.

Rheometry testing demonstrated that the stiffness (storage modulus) of the gels increased in dual crosslinked gels (117.6 ± 6.9) more than 5-fold from photochemically crosslinked gels (21.4 ± 1.8 Pa)(Fig. 2). Previous results, showing the lack of significant difference between the storage modulus of non-crosslinked, spontaneous collagen gels (25.8 ± 1.5 Pa) and photochemically crosslinked gels, were also confirmed as a t-test results in $p = 0.5814$ between these two groups.

We attribute the resulting increase in G' of dual-crosslinked hydrogels to the addition of new chemical crosslinks throughout the collagen matrix with a resulting increase in crosslinking density. The five-fold increase in G' by EDC/NHS on prior photocrosslinked hydrogels, in comparison to the null effect of EDC/NHS on spontaneously-formed collagen gels discussed earlier, is an interesting observation. A possible explanation is that photocrosslinking treatment provides a stabilizing effect to the hydrogel, aligning collagen molecules to make the EDC/NHS crosslinking more efficient. EDC/NHS crosslinking increases stiffness of structured tissues, such as amnion[151], tendon[165], and sheep dermis[166]. Therefore, forming a more organized collagen matrix by photocrosslinking before exposure to EDC/NHS can positively affect the G' of the hydrogel.

Any crosslinking paradigm for ultimate clinical use cannot be toxic to the encapsulated cells. It is clear that chondrocytes are viable after encapsulation in the dual-crosslinked matrix (Fig. 5). Encapsulation of chondrocytes in type I collagen hydrogels, both spontaneous and photocrosslinked, was shown to be non-toxic in previous studies. The dual-crosslinked constructs were shown to be 96 ± 2% viable, well above the threshold of 90% viability for good ECM generation capacity used in previous studies[75]. Dead cells were few in number and confined to the outer extremes of the construct. Cells also appeared to adhere well to the dual-crosslinked matrix.

The gels that underwent dual crosslinking also retarded degradation by collagenase digestion (Fig. 1). Protection from proteases is important for matrix stability during early-stage chondrocyte viability and ECM generation, but over time the initial matrix must be remodeled by natural enzymatic activity and deposition of new hyaline cartilage ECM. If not, the crosslinked type I collagen matrix will impede production of neocartilage. We anticipate that there will be an optimum crosslinking level that provides sufficient stiffness to the gel but also allows for gradual enzymatic digestion and remodeling *in vivo*. We have shown by using different concentrations of EDC/NHS or treatment times that we can fine-tune the stiffness and also the degradation rate. Although collagenase degradation is affected by exposing the hydrogel to even the most dilute EDC/NHS groups, there is no significant effect on storage modulus until the hydrogel is exposed to higher concentrations. Compared to full crosslinking (60 minutes exposure time and 33 mM EDC / 6 mM NHS) the stiffness of the gel increased at lower

concentrations and lower times of exposure (Figure. 3, 4). Further studies are planned to investigate the cartilage generation capacity of encapsulated chondrocytes in gels in these groups.

A major concern in articular cartilage engineering is the ability of a construct to withstand biocompressive forces in the knee, which can be up to 3.40 ± 0.18 times patient bodyweight during a normal walking gait[167]. The increase in mechanical properties after dual-crosslinking treatment was a very positive result. Creating a construct that has a higher storage modulus, (stiffer) would be beneficial as long as the increased stiffness does not impede neocartilage deposition within the matrix. Using a construct similar to those tested here may lead to shorter patient immobilization periods, shorter post-operative physical therapy periods, and an overall faster recovery when compared with recovery periods after solution-based cartilage reparative procedures like ACI. The proven viability of encapsulated chondrocytes and the protection against rapid enzymatic degradation that is provided by this dual-crosslinking paradigm may offer a route to a new, matrix-assisted articular cartilage replacement system.

CONCLUSIONS

From these data we can conclude that photochemically crosslinking collage gels increases their resistance to collagenase digestion two-fold. Adding a chemical crosslinking step to the photochemical crosslinking makes them even more resistant to digestion with collagenase without compromising the cell viability. The storage modulus of dual-crosslinked constructs was increased 5-fold over that of both photocrosslinked and spontaneous gels. Thus, the photochemical crosslinking, with or without chemical crosslinking, could resist degradation *in vivo* and be used as a scaffold for delivery of chondrocytes into cartilage defects. Changing the crosslinking strategy can improve the stiffness of the gel to provide additional stability to the gels when used *in vivo*.

ACKNOWLEDGEMENTS

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Chapter 6

Cartilage regeneration using different chondrocyte sources and photochemically crosslinked gelatin-methacrylamide (GelMA) hydrogel

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Manuscript in preparation.

ABSTRACT

Injectable engineered cartilage that maintains a predictable volume and produces comparable extracellular matrix formation would allow repair and regeneration of native cartilage tissue with minimally invasive techniques. In this study, we hypothesized that photochemically crosslinked gelatin-methacrylamide (GelMA) hydrogel would make suitable scaffolds for cartilage tissue engineering with favorable cell viability and ideal histological and biochemical properties. Swine auricular and articular chondrocytes were isolated and mixed with premade GelMA solution respectively, and irgacure 2959 was chosen as photo initiator for gel crosslinking using 365 nm UV light wavelengths. The final cell concentration was 40 X 10^6 / mL. Chondrocyte viability was measured 24 hours after initial gelation using Live/Dead assay. After 2 weeks precultrue in the incubator, 6-8 samples (cylinder shape, 0.6 cm diameter and o.4 cm height) per group were implanted into nude mice for each time period (6 and 12 weeks). The dimensions of each specimen were recorded before implantation and after explantation. Histological and biomechanical analyses were performed. Photochemically crosslinking GelMA hydrogel permitted more than 80% cell viability 24 hours after irradiation. At each explantation time point, the diameters of the constructs in GelMA/articular chondrocyte were significantly lower than that of the constructs in GelMA/auricular chondrocyte group. Histological evaluations (n=3) indicated that new cartilaginous matrix was synthesized by the transplanted chondrocytes in all experimental groups, and no significant morphological differences were observed in histological staining of the matrix and the cellular organization between the two different experimental groups. For better interpretation, glycosaminoglycan (GAG) content was normalized against total DNA content for biochemical analyses (n=4-5). At each explantation time point, the value of GAG/DNA in GelMA/articular chondrocyte group is statistically lower than that in GelMA/auricular chondrocyte group. The results of this study suggest that this novel biomimetic gelatin-methacrylamide (GelMA) hydrogel scaffold provides a favorable microenvironment for both articular and auricular chondrocytes to produce extracellular matrix. The engineered neocartilage is similar to native cartilage histologically, and biochemically. It was possible to engineer injectable cartilage with chondrocytes from different sources, resulting in neocartilage with slightly different properties.

INTRODUCTION

Advances in tissue engineering in the past two decades have rekindled orthopaedic surgeons' and plastic surgeons' interest in the possibility of generating tissue from cultured cells in situ, for reconstruction or augmentation. A potentially limitless supply of functional tissue could be engineered by expanding cells from a small amount of donor tissue *in vitro*. The successful use of tissue engineering techniques to form engineered cartilage would allow the reconstruction of musculoskeletal or craniofacial tissue deformities without the complications associated with prosthetic materials, allografts, or large autografts. Furthermore, minimally invasive cartilage implantation for use in surgical procedures would be possible if tissue engineered cartilage could be produced in an injectable moldable form and could sustain its volume for prolonged periods.

Functional biological prostheses have been created through tissue engineering by suspending dissociated cells in biodegradable polymer scaffolds, leading to new tissue formation[168]. A gel-like polymer scaffold is essential for engineering injectable cartilage for minimally invasive implantation. Two kinds of injectable polymers have been widely used in tissue engineering, namely, naturally derived polymers such as fibrin glue polymer[57], alginate[64], collagen gel[169], and agarose gel[170] and synthetic polymers such as poly(ethylene oxide) hydrogel[171], dimethacrylate poly(ethylene oxide)[172], poly-(propylene fumarate-co-ethylene glycol), and Pluronic 127 (polyethylene oxide and polypropylene oxide)[173]. An ideal injectable polymer should be nonreactive, maintain a three dimensional configuration, and allow for extracellular matrix deposition. Moreover, it should have well-defined and controllable degradation properties that permit tissue formation[62].

Elisseeff *et al*[174] were the first to report a process for photopolymerizing poly(ethylene oxide) (PEO) into a hydrogel network to encapsulate chondrocytes for cartilage matrix formation. Chondrocyte photoencapsulation is advantageous due to the ease of filling irregularly shaped defects, which leads to good contact between the tissue-engineered construct and the surrounding native tissue. This technique allows for the noninvasive implantation of chondrocyte-polymer constructs by exposing prepolymer solutions containing cytocompatible photoinitiators to low-intensity visible or ultraviolet light. These efforts have focused primarily on photopolymerizable hydrogels based on poly(ethylene glycol) (PEG), PEO, and hyaluronic acid[150, 174-177]. Because PEG and PEO are long carbon chain polymers, they can be chemically modified to control hydrogel degradation and enhance extracellular matrix distribution in the engineered tissue. Recently, our group has been exploring novel photochemically crosslinked gelatin methacrylamide (GelMA) hydrogels as cell carrier for cartilage regeneration. GelMA hydrogels are enzymatically degradable and tunable for specific regenerative applications through modification of the degree of methacrylation and the polymer concentration. They are shown to support chondrocyte viability and differentiation and give wide ranging mechanical properties depending on several cross-linking parameters[178]. It could be a suitable substrate, given the extensive cartilage matrix formation we previously observed in this gel[179].

Sustaining the volume of injectable engineered cartilage with time is critical for clinical application of these techniques. Based on our experience, the change of shape and volume of *in vivo* hydrogel constructs might be inevitable as time goes by. However, any tremendous volume reduction or overgrowth of injectable engineered cartilage would undermine the success of reconstructions. Our previous studies demonstrated that injectable engineered cartilage produced with articular chondrocytes and fibrin glue polymer underwent substantial reductions in mass and volume after implantation[57, 74]. This study investigated how well engineered cartilage produced with different cell sources including articular and auricular chondrocytes and novel photochemically crosslinked GelMA hydrogels conserved its volume, as well as evaluating the viability of cells encapsulated into this biomimetic GelMA hydrogels, the histological and biochemical properties of the new matrix.

MATERIALS AND METHODS

Preparation of GelMA

GelMA was prepared by reaction of type A gelatin (Sigma–Aldrich, St. Louis, Missouri, USA) with methacrylic anhydride (Sigma–Aldrich) at 50 ^{0}C for 1 h. Briefly, methacrylic anhydride was added dropwise to a 10% solution of gelatin in phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, California, USA) under constant stirring. To achieve a high degree of functionalization (DoF), 0.6 g of methacrylic anhydride was added per gram of gelatin. The functionalized polymer was dialyzed against distilled water for 3 d at 40 ^{0}C to remove methacrylic acid and anhydride, neutralized with 10% sodium bicarbonate (Merck, Darmstadt, Germany), freeze-dried and stored at 20 ^{0}C before use. The DoF was determined as previously described, by using ninhydrin to quantify the concentration of residual free amine groups in the synthesized GelMA relative to the starting material[178].

Chondrocyte-polymer constructs

Auricular and articular cartilage was harvested from 3- to 6-month-old Yorkshire pigs. Chondrocytes were isolated with collagenase digestion in a 37 °C reciprocal shaking water bath for 12 to 18 hours. Auricular cartilage was digested in 0.2% collagenase (Worthington Biochemical Corp., Lakewood, N.J.) and 1% antibiotic/antimycotic solution (10,000 units/mL penicillin, 10 mg/mL streptomycin, and 25 g/mL amphotericin B in 0.9% sodium chloride; Sigma Chemical Co., St. Louis, Mo.), whereas a 0.1% collagenase solution was used for articular cartilage digestion. After digestion, the solution was filtered through a 100 μm nylon filter to remove the undigested fragments. The cell suspension was centrifuged at 1500 rpm for 10 minutes. The cell pellet was washed twice in phosphate-buffered saline (Sigma) with 2% antibiotic/ antimycotic solution. The viability of the chondrocytes was assessed with trypan blue staining and was recorded as a percentage of viable chondrocytes per high-power field. Only chondrocyte suspensions with a viability score of 90 percent or greater were used in the experimental studies described below. The exact cell count per milliliter was established with a hemacytometer.

The light initiator of irgacure 2959 (BASF Corp., Florham Park, NJ) was dissolved into the sterile PBS at the concentration of 1.25mg/mL. The solution was well mixed on the shaker at 70 °C until the irgacure was completely dissolved and then got cooled down. The GelMA powder made as previously described was dissolved into the irgacure-supplemented sterile PBS at 37 ^{o}C to obtain the concentration of 12.5%. Chondrocytes were suspended in sterile PBS at the concentration of 200 X 10^6/mL of 20% of final volume, and then the cell-PBS suspension was added to premade irgacure-supplemented GelMA solution. The chondrocyte-GelMA constructs were made in bone wax wells created by 6mm biopsy punches measuring in 6 mm diameter and 4 mm height. All of the specimens were irradiated under 365 nm wavelength UV devices for 15 minutes. After crosslinking, all of the specimens were precultured in the incubator for up to 2 weeks. At 24 hours, 3 samples from each experimental group were tested for cell viability using Live/Dead assay. 16 specimens of each cell type were implanted into dorsal subcutaneous pouches in nude mice. Eight specimens of each cell type were harvested at 6 and 12 weeks, and the specimens were tested through histological and biochemical analyses.

Viability analysis

Cell viability was visualized 24 hours after irradiation for each group (n=3-4) using Live/Dead cell viability/cytotoxicity assay kit (InvitrogenCorp.,Carlsbad,CA).According to manufacturer's instruction, 8 mM of calcein AM and 4 mM of ethidium homodimer-1 were added to the constructs, and the constructs were incubated for 20min at 37 ^{0}C. Cell viability was examined using a fluorescence microscope (Nikon Inc., El Segundo, CA). Live cells emit green fluorescence when viewed under excitation at 494nm and dead cells emit red fluorescence when viewed under 528nm. Images of 5 separate randomly chosen fields of view were taken for each sample under both wavelengths.

Histological analyses

Three randomly selected specimens from each group at each time point were placed in 10% phosphate-buffered formalin for 24 hours, embedded in paraffin, and sectioned. Cross-sections were prepared from a minimum of two areas from each specimen and were stained with hematoxylin and eosin. Other sections were stained with Safranin-O, to verify the production of sulfated proteoglycans. The sections were evaluated in a blinded manner by three independent reviewers, to determine the presence or absence of cartilage formation.

Biochemical evaluations

Sample preparation

One 30- to 40-mg piece of engineered cartilage tissue was collected from each specimen. The pieces were weighed, lyophilized for 24 hours, and weighed again. The difference between the weights before and after lyophilization was calculated as the water content of the specimen (data not shown). These samples were then subjected to biochemical analyses to determine the glycosaminoglycan and DNA contents, which are reported as percentages of the unit weight of dry tissue. Briefly, the specimens were digested with the addition of 1.0 mL of solution containing 100 mM sodium phosphate, 10 mM sodium ethylenediaminetetraacetic acid/disodium salt/dihydrate, 10 mM cysteine hydrochloride (Sigma), 10 mM ethylenediaminetetraacetic acid (BDH, Poole, Dorset, England), and 125 g/mL papain (Sigma). The specimens were incubated in a 60 °C water bath for 24 hours[180].

Glycosaminoglycan contents

The glycosaminoglycan contents of tissue digests were quantified according to a previously described method[180]. Briefly, 10 µl of papain digest was added to 200 µl of 1,9-dimethylmethylene blue dye (pH 3.0), and absorbance at 525 nm was measured with a spectrophotometer immediately after addition to the dye. Glycosaminoglycan contents of the specimens were determined by using chondroitin 6-sulfate from shark cartilage (Sigma) as a standard. All samples and standards were analyzed in duplicate. Glycosaminoglycan contents were measured in micrograms per milliliter and reported as a percentage of dry tissue weight.

DNA contents

The DNA contents of the tissue digests were quantified with a previously described method[181]. The DNA contents of the specimens were determined by measuring the fluorescence (360/465 nm) of aliquots immediately after mixing with bisbenzimidazole dye (Hoechst 33258; Hoefer Scientific Instruments, San Francisco, Calif.). The dye was used at a concentration of 0.1 µg/mL in 10 mM Tris, 1 mM ethylenediaminetetraacetic acid, 0.1 mM NaCl. DNA contents of the specimens were determined by using calf thymus DNA (Sigma) as a standard. All samples and standards were analyzed in duplicate. DNA contents were measured in micrograms per milliliter and reported as a percentage of dry tissue weight.

Statistical Analyses

Two-way analysis of variance was performed with SigmaStat 2.03 software (version 2.03; SPSS, Inc., Chicago, Ill.), to compare the data on dimension, mass, biochemical values, and biomechanical values (mean SD) among experimental groups. A Bonferroni post hoc test was performed, which corrects for multiple testing. Significance was taken as $p < 0.05$ or as otherwise indicated.

RESULTS

Cell viability

Chondrocytes exhibited more than 80% viability in both GelMA/articular chondrocyte group and GelMA/auricular chondrocyte group (Fig. 1). There's no obvious difference between the two experimental groups.

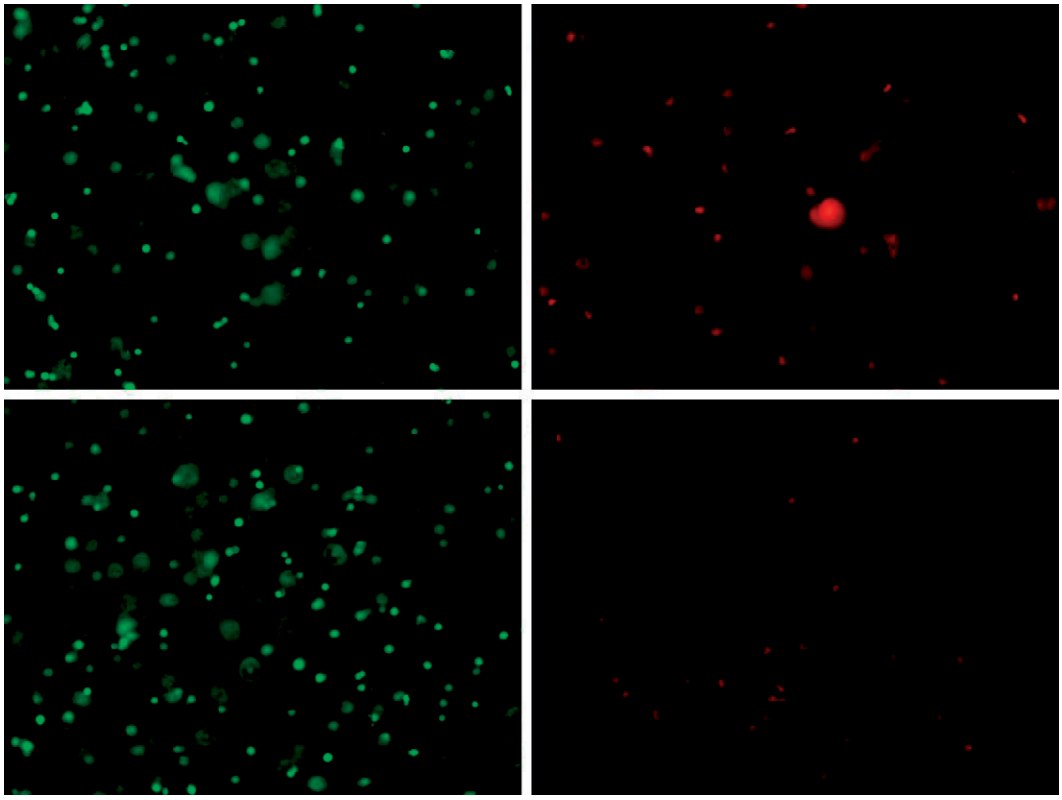


Figure 1. Fluorescent staining (live/dead assay) of encapsulated cells in the crosslinked GelMA hydrogel 24 hours after irradiation using UV light source (365 nm, 15 minutes). Fluorescent green spots (left) indicate living cells and red spots (right) indicate dead cells. Top row indicates the GelMA/articular chondrocyte group, and the bottom row indicates GelMA/auricular chondrocyte group. (200 X)

Changes in size

The diameters of all constructs in GelMA/articular chondrocyte group decreased at both 6 and 12 weeks *in vivo*. However, the diameters of all constructs in GelMA/auricular chondrocyte group increased at both 6 and 12 weeks *in vivo*. At each time point, the diameters of the constructs in GelMA/articular chondrocytes were significantly lower than that of the constructs in GelMA/auricular chondrocyte group (Fig. 2).

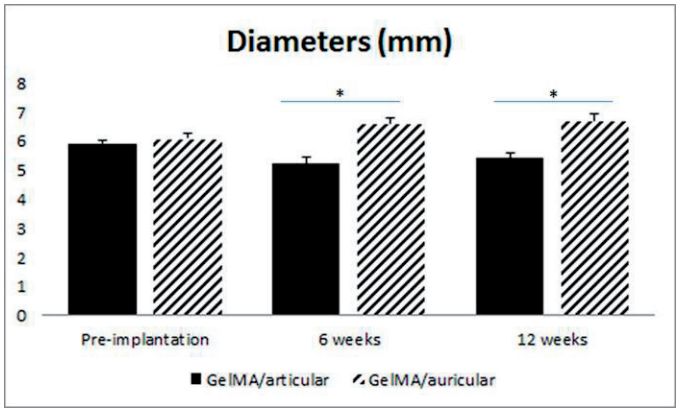
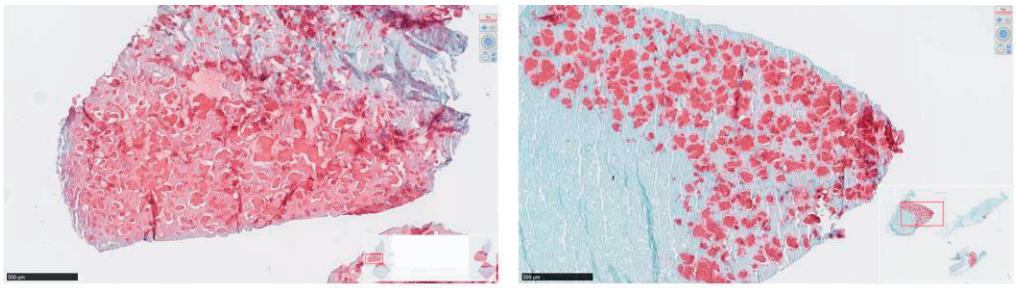


Figure 2. Dimensional changes with respect to time (*, $P<0.05$, n=4).

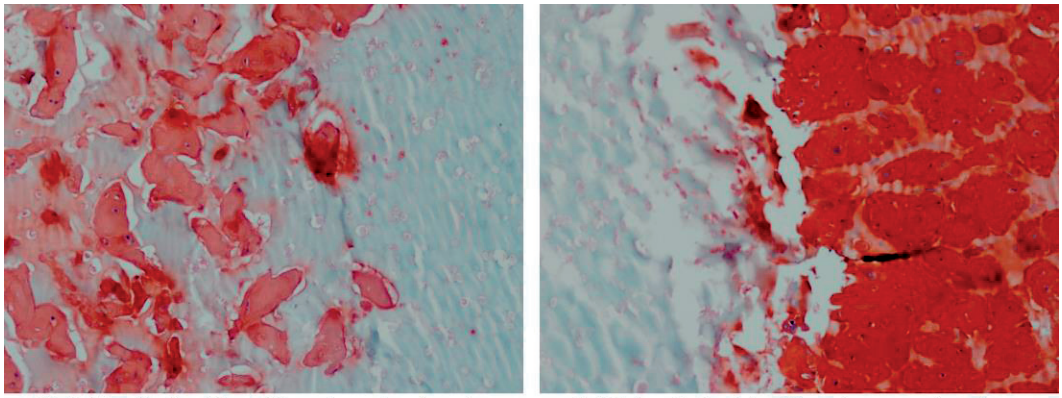
Histological analysis

Figure 3 and 4 show that new cartilaginous matrix was synthesized in all harvested groups (both GelMA/articular chondrocyte group and GelMA/auricular chondrocyte group) at 6 and 12 weeks from nude mice. The morphology of the neocartilage appeared similar to respective native cartilage, with cells located within typical chondrocyte lacunae and surrounded by cartilaginous matrix. Deposition of glycosaminoglycan was also evident by Safranin-O staining. There's also remnant of GelMA hydrogel observed at each time point for both experimental groups. No significant morphological differences were observed in histological staining of the matrix and the cellular organization between the two different experimental groups. No vascular in-growth was observed in any of the constructs (Fig. 3 & 4).



GelMA + Swine Knee Chondrocytes (6w) GelMA + Swine Ear Chondrocytes (6w)

Figure 3. Safranin-O staining of engineered neocartilage tissue (6 weeks from nude mice, 50 X).



GelMA + Swine Knee Chondrocytes (12w) GelMA + Swine Ear Chondrocytes (12w)

Figure 4. Safranin-O staining of engineered neocartilage tissue (12 weeks from nude mice, 100 X).

Biochemical analyses

For better interpretation, glycosaminoglycan content was normalized against total DNA content. At 6 weeks, the amount for GelMA/articular chondrocyte group was 13.5±0.7 µg glycosaminoglycan/ µg DNA, and the amount for GelMA/auricular chondrocyte group was 17.1±0.6 µg glycosaminoglycan/ µg DNA. At 12 weeks, the amount for GelMA/articular chondrocyte group was 12.7±0.4 µg glycosaminoglycan/ µg DNA, and the amount for GelMA/auricular chondrocyte group was 15.2±0.5 µg glycosaminoglycan/ µg DNA. At each time point, the amount of GelMA/articular chondrocyte group was significantly lower than that of GelMA/auricular chondrocyte group (Fig. 5).

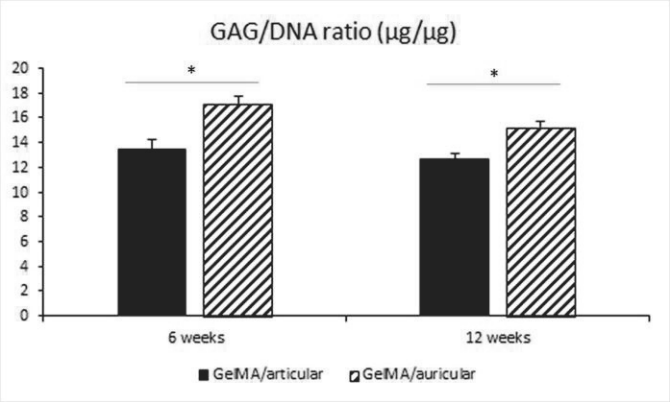


Figure 5. Biochemical analyses. The changes of the ratio of glycosaminoglycan to total DNA amount with respect to time (*, $P<0.05$, n=6).

DISCUSSION

Numerous scaffolding materials have been used for cell delivery in cartilage regeneration. Scaffolds provide a three-dimensional environment that is desirable for the production of cartilaginous tissue. Ideally the scaffold should: 1) have directed and controlled degradation, 2) promote cell viability, differentiation, and ECM production, 3) allow for the diffusion of nutrients and waste products, 4) adhere and integrate with the surrounding native cartilage, 5) span and assume the size of the defect, and 6) provide mechanical integrity depending on the defect location[182]. Hydrogels have been extensively applied in the research field of tissue engineering and regenerative medicine because of their three dimensional (3D) nature, high water content and wide range of polymers that can be used for their fabrication. Besides, development of suitable injectable hydrogel materials would permit minimally invasive techniques for cartilage repair and regeneration. This study demonstrated the feasibility of neocartilage formation applying different chondrocyte sources (articular and auricular) encapsulated into a novel biomimetic GelMA hydrogel material.

A significant problem of spontaneous hydrogels is the plasticity and their propensity to deform under even mild compressive forces. These gels are easily deformed by the application of external compressive forces and cannot be restored to their original state[75]. To advance hydrogel-based biomaterials, a wide variety of stimuliresponsive hydrogels have been developed to control their properties with external stimuli (e.g. cross-link density, hydrophobicity, swelling rate, permeability, degradability and mechanical strength)[183]. Among these stimuli, light is a particularly interesting option as it is a remote stimulus that can be controlled both spatially and temporally with great ease and convenience[184-188]. Our group has successfully demonstrated chondrogenesis using photochemically crosslinked collagen gel[75] and poly(ethylene glycol) gel[115]. In this most recent study, we were focused on photochemically GelMA hydrogels for cartilage regeneration

The Live/Dead assay result showed a satisfying viability of cells encapsulated into this GelMA hydrogel 24 hours after photochemically crosslinking, which would be the first important premise for further cartilage regeneration. The results of this study demonstrated that there were statistically significant differences in diameters between the specimens composed of two different chondrocyte sources. In agreement with the results reported by Xu *et al*.[62], the volume of specimens made with articular chondrocytes and hydrogel decreased significantly throughout the experimental period. When auricular chondrocytes were used in this experiment, there was a tendency toward overgrowth. Interestingly, the biochemical data showed that the ratio of glycosaminoglycan to total DNA of GelMA/auricular chondrocyte group was significantly greater than that of GelMA/articular chondrocyte group, which is consistent with the findings of diameter change. These findings demonstrate that, in this system, the chondrocyte source has a significant effect on the volume of

tissue-engineered cartilage. These special properties should be considered in its future application for further studies.

It could be argued that these results are not applicable to the human situation, because the subcutaneous environment of athymic mice is unlike the submuscular environment of the human face. Certainly this could be true for constructs in which articular chondrocytes are used, because the subcutaneous environment is quite different from the intact diarthrodial joint. However, vascular invasion was not noted in any specimen. We agree that the model may have some deficiencies, but the nude mouse is a suitable small animal model to pilot test new scaffolds and tissue formation. In many ways they can be considered a "living" petri dish for studying cartilage matrix formation from cell-seeded scaffolds. Therefore, this study still could provide illuminating information that may influence the choice of chondrocyte source for different purposes of cartilage repair and regeneration either in orthopaedic surgery or plastic surgery.

The histological results from this study demonstrated that this GelMA scaffold provided a favorable environment for both auricular and articular chondrocytes to secrete new cartilage matrix. All of the specimens showed cartilage morphology with chondrocytes located within isolated lacunae surrounded by matrix. There's no significant difference between articular chondrocyte group and auricular chondrocyte group. Hypercellularity of new matrix is commonly observed in engineered cartilage[62, 75, 115]. Although we could still notice that some remnants of the hydrogel were present at 12 weeks after implantation. Based on previous findings[178], crosslinking GelMA could enhance the strength and stiffness of this hydrogel. We believe that this GelMA hydrogel has a better mechanical property and could last a relatively longer time compared to other common used hydrogel candidates including fibrin glue *etc*. to support the encapsulated cells forming more cartilaginous matrix. We assume that hydrogel remnants will finally be replaced by neotissue in the long term.

Photochemical crosslinking relies on the production of radical oxygen species. However, one limitation of this technique is the potential for producing free radicals that could damage and kill the embedded cells. Adding in radical scavenger molecules could reduce cell damage due to the radicals. Cho *et al[189]* reported that antioxidants, such as catalase and salicylic acid, protected human fibroblasts from the cytotoxic effect of hydrogen peroxide produced by illumination of ribloflavin. The addition of antioxidants could therefore allow higher concentrations of riboflavin or longer exposure times to be used in order to obtain stronger mechanical properties of the gel that might perform better *in vivo*. How to control and manipulate this photochemically crosslinked GelMA hydrogels more exquisitely *in vivo*, especially in large immunocompetent animal models, will be our focus in the next phase.

CONCLUSIONS

The results of this study suggest that this novel biomimetic gelatin-methacrylamide (GelMA) hydrogel scaffold provides a favorable microenvironment for both articular and auricular chondrocytes to produce extracellular matrix. The engineered neocartilage is similar to native cartilage histologically, and biochemically. It was possible to engineer injectable cartilage with chondrocytes from different sources, resulting in neocartilage with slightly different properties.

ACKNOWLEDGEMENTS

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Chapter 7

Porous poly(vinyl alcohol)-hydrogel matrix-engineered biosynthetic cartilage

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ABSTRACT

The objective of this study was to fabricate hydrogel matrix-engineered biosynthetic cartilage using a porous poly(vinyl alcohol) hydrogel (PVA-H) and articular chondrocytes. Chondrocytes were suspended in fibrin gel (FG) or saline carriers and injected into porous PVA-H discs and three-layered constructs (PVA-H between devitalized cartilage). After implantation in nude mice, PVA discs were explanted at 6 weeks and subjected to creep testing for a 20 h period. The three-layered constructs were explanted at 12 weeks and subjected to tensile testing to determine the strength of the interface between the engineered hydrogel and devitalized cartilage. Histological analysis revealed PVA-H porous channels occupied by chondrocytes. Extracellular matrix was identified by Safranin-O and Toluidine Blue stains. Immunohistochemical analysis revealed a positive stain for COL II and scant staining for COL I. Creep and relaxation response of PVA-FG-chondrocyte constructs was similar to that of native cartilage. The presence of cells and FG significantly enhanced the integration strength of layered constructs ($p<0.05$). These results demonstrate that porous PVA-H in combination with FG and chondrocytes provides a favorable microenvironment for tissue engineering of articular cartilage, creating a biosynthetic construct that can adhere to native devitalized articular cartilage utilizing hydrogel matrix-engineered technology.

INTRODUCTION

Articular cartilage lesions due to acute and chronic injuries require a complex set of morphological, biomechanical, and biochemical factors necessary for repair that often result in only suboptimal joint architecture and can lead to osteoarthritis. Treatments such as osteochondral allografts, microfracture technique, osteoarticular transfer system, and autologous chondrocyte implantation are surgical modalities to resolve pain and restore function. However, the long-term postoperative histological findings of these procedures often do not resemble the appearance of native nonarthritic hyaline cartilage and the resulting inferior tissue may not be durable over the long term. The limited regenerative characteristics of cartilage have led to the synthesis of biomaterials that mimic the properties of this tissue, among them, hydrogels[190].

Hydrogels are favorable scaffolds for engineering neocartilage. Not only do they possess unique properties that mimic a three-dimensional environment similar to that of native tissues, but also innumerable modifications can be addressed through crosslinking, annealing, and changes in pH and temperature. Further, copolymer hydrogel blends permit a vast array of formulations that are applicable in multiple areas of tissue engineering, making these materials ideal scaffolds for encapsulating chondrocytes, inserting them into a defect by means of minimally invasive techniques, and engineering neocartilage in situ. Our group has previously described the use of hydrogels, including solubilized collagen, fibrin gel (FG), hyaluronic acid, and sodium alginate, to engineer neocartilage *in vivo*[57, 75, 176, 191]. However, compared to native articular cartilage, these hydrogels are mechanically fragile after crosslinking and would therefore be unable to support physiological joint load before extracellular matrix (ECM) deposition occurs. To provide an adequate structural architecture similar to that surrounding the cartilaginous lesion, newly implanted hydrogels may require an additional nondegradable matrix to support the biomechanical forces distributed throughout the articular joint. A nondegradable porous poly(vinyl alcohol)-hydrogel (PVA-H) could function as an additional structurally adequate matrix for mechanically weak hydrogels such as FG.

PVA can be synthesized into porous hydrogel formulations that demonstrate a high degree of swelling in aqueous environments. This inert, nontoxic, biocompatible, and biomimetic material can be tailored to be viscoelastic and have high water content[192, 193]. These properties are ideal for simulating the mechanics of native cartilage tissue. Further, it can be molded into any size or shape, which is ideal for filling irregularly sized or shaped cartilaginous defects. We have previously investigated and described how PVA-acrylamide hydrogels possess characteristics similar to load-bearing cartilage, due in part by noncollapsible pores filled with water throughout the gel[193]. We have also studied the interaction between a porous PVA-H in combination with sodium alginate hydrogel and human nasoseptal chondrocytes, engineering neocartilage *in vivo* and demonstrating the feasibility of combining two hydrogels with very distinct mechanical properties[191]. The previously mentioned attributes make a porous PVA-H an ideal candidate for biosynthetic cartilage implantation. In this study, we hypothesized that articular chondrocytes suspended in either FG or saline could be combined with a porous PVA-H, creating a biosynthetic construct that could further mimic the histological, immunohistochemical, and biomechanical properties of native hyaline cartilage after *in vivo* implantation.

MATERIALS AND METHODS

Cartilage harvest, chondrocyte isolation, and expansion

All actions were approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital. After euthanasia, knee joints from 3-month-old Yorkshire swine were collected from the operating room, taken to the laboratory, and submerged in povidone-iodine solution for 30min. Aseptically, each joint was dissected, knee capsules were opened, and a dermal punch biopsy was used to harvest cartilage disks measuring 6mmin diameter and 1.5mm in thickness. The

remaining articular cartilage was harvested using a No. 10 scalpel blade and manually minced into 1mm^3 pieces using razor blades. The minced tissue was placed in 50mL polypropylene tubes and digested for 18 h at 37^{0}C using 0.8% collagenase type II (Worthington Biochemical Corporation). After digestion, chondrocytes were rinsed and washed twice with phosphate-buffered saline (PBS). Cell number and viability superior to 95% was determined using a hemocytometer and the trypan blue dye test. To increase cell numbers, aliquots of 1.5 x 10^6 chondrocytes were placed in 150 cm^3 cell culture flasks with cell media containing HAM's F-12 medium with L-glutamine supplemented with 10% fetal bovine serum, 50U/mL penicillin, 50 mg/mL of streptomycin, 50mg/ML ascorbic acid, and 0.1mM nonessential amino acids mix at 37^{0}C and 5% CO2. Medium was changed every third day until 90% confluency was achieved. Enzymatic digestion with 0.05% trypsin EDTA was used to collect cells from the flasks.

PVA hydrogel preparation

PVA-H was prepared as previously described[193]. Briefly, porous PVA-H was prepared by theta gelation by dissolving PVA (115,000 g/mol; Scientific Polymer Products) and polyacrylamide-co-acrylic acid (PAAm-co-AAc; Sigma- Aldrich) and poly(ethylene glycol) (PEG; Acros Organics) in deionized (DI) water at 90^{0}C. For gelation, the resulting solution was molded into sheets and cooled down at 25^{0}C for 24 h. The gel was immersed in DI water for equilibrium and subjected to electron beam sterilization.

Environmental scanning electron microscopy imaging

Detailed imaging was performed on the synthesized PVAH to determine the structural architecture and porosity. Briefly, PVA-H was exposed to freeze fracture in liquid nitrogen and subsequent rehydration in DI water. Using 5 kV accelerating voltage, images were acquired with an FEI/ Philips XL30 environmental scanning electron microscope-field emission gun. PVA-H architecture was magnified 250 and 1200 times on the freeze-fractured surfaces.

Construct preparation

Disc model. PVA gel discs (n¼6 for each group) were cut from a sheet of PVA-H using a 6mm dermal biopsy punch. Final construct dimensions were 2.1mm height and 6mm in width. Groups for this study were the following:

(A) PVA + 0.9% saline

(B) PVA + FG

(C) PVA + FG + chondrocytes

(D) PVA + 0.9% saline + chondrocytes

FG was created by mixing equal volumes of bovine fibrinogen (80 mg/mL) and thrombin (50 U/mL) solutions. Using a 25-gauge needle, constructs from groups A–D were injected with a volume of 40 mL of their designated solution. Cell concentration for groups C and D was 40 X 10^6/mL.

Three-layered construct model. A three-layered construct model was assembled as previously described by our group[65]. The same groups assembled for the disc model were also assembled for the three-layered constructs. For this model, swine articular cartilage discs that were punched from the femoral condyles using a 6mm dermal biopsy punch were devitalized by freeze–thaw cycles. The devitalized cartilage was dried using sterile gauze. Any subchondral bone that was still present in the tissue was removed using a razor blade. Then, the PVA-H was placed between the devitalized cartilage, creating a three-layered structure. A 5-0 nonabsorbable, sterile surgical monofilament suture was used to secure the three layers in place and was removed after the *in vivo* period.

Construct implantation

Male nude mice (COX-7 Laboratories, Massachusetts General Hospital, Boston, MA) were obtained at 6 weeks of age and allowed to acclimatize for 1 week. Intraperitoneal tribromoethanol (400mg/kg) was used for inducing anesthesia. A 2 cm midline incision was made on the dorsum under sterile conditions. Blunt dissection was achieved using a Stevens tenotomy scissor, creating four

subcutaneous pockets. Four constructs were implanted in every mouse. The incision was closed using stainless steel Autoclip staples (Becton Dickenson) and removed after 14 days. PVA disc constructs were harvested after 6 weeks, whereas the three-layered constructs were harvested after 12 weeks.

Histological analysis

Two samples from each group were selected for the histological analysis. Specimens were fixed in 10% phosphate-buffered formalin for 24 h, dehydrated in increasing concentrations of ethanol, and embedded in paraffin. Serial 5 mm sections were obtained. Before staining with hematoxylin and eosin, Safranin-O, and Toluidine Blue, slides were deparaffinized using xylene and absolute, 95%, and 70% ethanol. A Nikon Eclipse E600 microscope was used to examine the slides, and obtained images were recorded with an Olympus DP25 color digital camera.

Immunohistochemical analysis

Two samples from each group were selected for the immunohistochemical analysis. Slides were stained by means of immunohistochemistry for the identification of collagen type I and II (COL I and COL II) deposition. Briefly, deparaffinized slides were treated with 2% bovine testicular hyaluronidase. 0.3% Hydrogen peroxide in methanol was applied and 10% goat serum was added to each slide. On separate slides, the primary antibodies for COL II (Chondrex, Inc.) and COL I (Abcam) were applied for 1 h at room temperature. Both antibodies were diluted 1:1000 in 1% bovine serum albumin in PBS. For negative control, N-Universal Negative Control was applied. The secondary antibody (horseradish peroxidase-labeled polymer) was added to the slides and 3,three-dimensionaliaminobenzidine was applied to each slide. Cell nuclei were counterstained with hematoxylin.

Creep testing

The creep behavior of the PVA disc construct model was evaluated immediately after explantation as previously described[193]. Five samples were tested for each group. Briefly, all samples were equilibrated at 40^0C in DI water before creep testing. The total creep strain (TCS) of the samples was determined on a custom-made mechanical tester in DI water at 40^0C by applying a 0.5MPa compressive stress for 10 h followed by a 10 h relaxation period under 0.05MPa compressive stress. Time, displacement, and load were recorded every 2 s during the 20 h period. The TCS was determined by averaging the values for each and expressed as a percent. This value was taken as the representative response to creep deformation for each of the different construct groups. Additionally, freshly harvested native swine articular cartilage was tested and compared to the engineered samples.

Integration strength

The three-layered constructs were tested to assess the mechanical integration between native and engineered tissue as described previously[60]. Five samples were tested for each group. Briefly, samples were patted dry with tissue paper to remove any excess moisture and were attached to circular polycarbonate rods using ~ 20 mL of cyanoacrylate glue. The rods, attached with the constructs, were mounted onto an EnduraTEC mechanical testing platform (Bose Corporation). About 0.2mL of PBS was applied on the exposed unattached construct surface to prevent dehydration. Constructs were pulled to failure in tension at a displacement rate of 10 mm/s, with resultant loads recorded at 5Hz. The applied tensile displacement and measurement of the tensile load generated a stress–strain curve. From this curve, the ultimate tensile strength (σ_{UTS}), energy to failure (E_f), Young's modulus (E_x), and the failure strain (ε_f) were determined. To simplify the presentation of numerical values, data were plotted into bar graphs using Microsoft Excel.

Statistical analysis

For the integration strength tests, a two-factor analysis of variance with post hoc Tukey test was performed on all mechanical properties to determine the significance between groups. Statistical significance was set at $p<0.05$.

RESULTS

Environmental scanning electron microscopy PVA-H observation

Environmental scanning electron microscope revealed a highly porous three-dimensional internal architecture composed predominantly of two structures, open channels and smaller cavities trapped throughout the PVA-H. The channels measured between 60 and 250 mm (Fig. 1A). These multiple interconnected channels created a communicating network throughout the engineered hydrogel. The smaller cavities resembled a honeycomb-like pattern (Fig. 1B). The overall structural composition of this scaffold consisted of an interconnected porous structure that allowed for the seeding of chondrocytes.

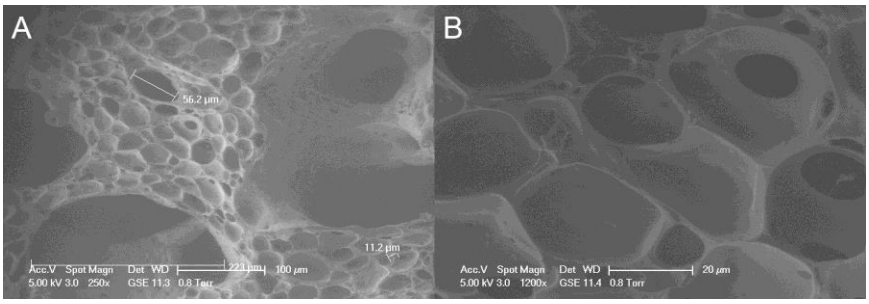

Figure 1. Environmental scanning electron microscopy images. A highly porous poly(vinyl alcohol) hydrogel (PVA-H) matrix was engineered. Cross section revealed interconnected channels (A). Close examination demonstrated a honeycomb-like pattern of the trapped cavities distributed throughout the hydrogel (B).

Gross examination, histological, and immunohistochemical analysis

Disc model. All constructs were recovered at the time of explanation. No signs of inflammatory reactions of the overlying soft tissue, edema, or—most importantly— extrusion were noted during the *in vivo* period or at the time of harvest. As expected with a nondegradable hydrogel, the original dimensions of all of the constructs were maintained during the *in vivo* period. Light microscopy showed porous regions to be occupied by neocartilage tissue. Hematoxylin and eosin staining did not reveal any neotissue on acellular constructs from groups A and B. Group C, containing chondrocytes suspended in FG, was characterized by neocartilage distributed throughout the open channels of the PVA-H matrix. ECM deposition was confirmed by Safranin-O and Toluidine Blue staining (Fig. 2A, B). Group D, containing chondrocytes suspended in saline, exhibited the same pattern of neocartilage distribution; however, ECM deposition staining was less intense (Fig. 2C, D). Immunohistochemical staining for COL II in group C revealed a dark brown homogenous stain (Fig. 3A), but staining for COL I was scant (Fig. 3B). Compared to group C, the staining for COL II in group D was less intense (Fig. 3C). In both groups C and D, the neocartilage was in close contact to the PVA-H, creating a tight interface.

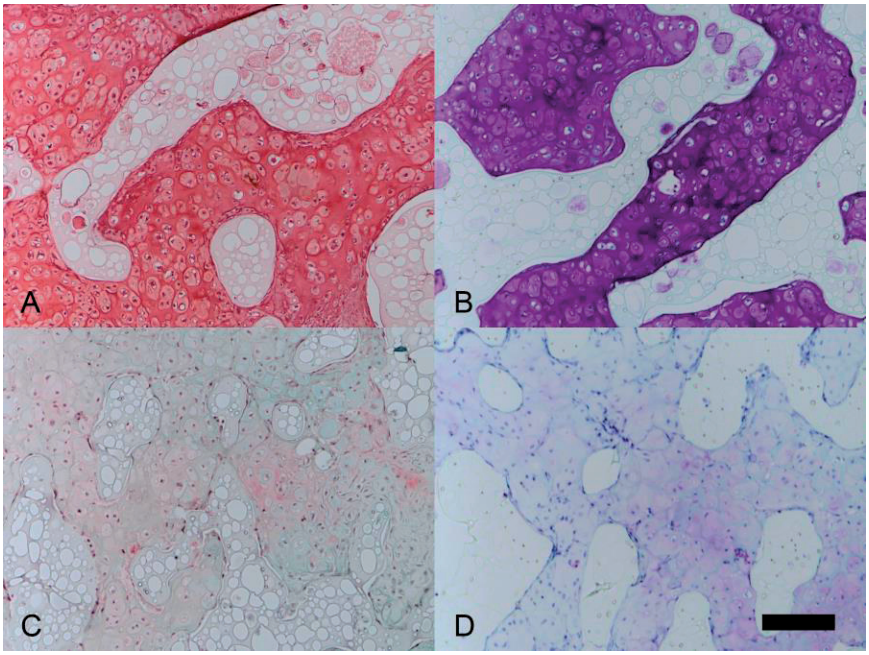

Figure 2. Representative Safranin-O (A, C) and Toluidine Blue (B, D) histological staining patterns for the group of constructs seeded with articular chondrocytes. (A, B) An intense stain for extracellular matrix deposition in PVA-H constructs seeded with articular chondrocytes suspended in fibrin gel (FG). A substantially less intense stain was found in the group of constructs seeded with chondrocytes suspended in saline (C, D). The unstained elements represent the porous PVA-H scaffold matrix. Scale bar: 200 mm.

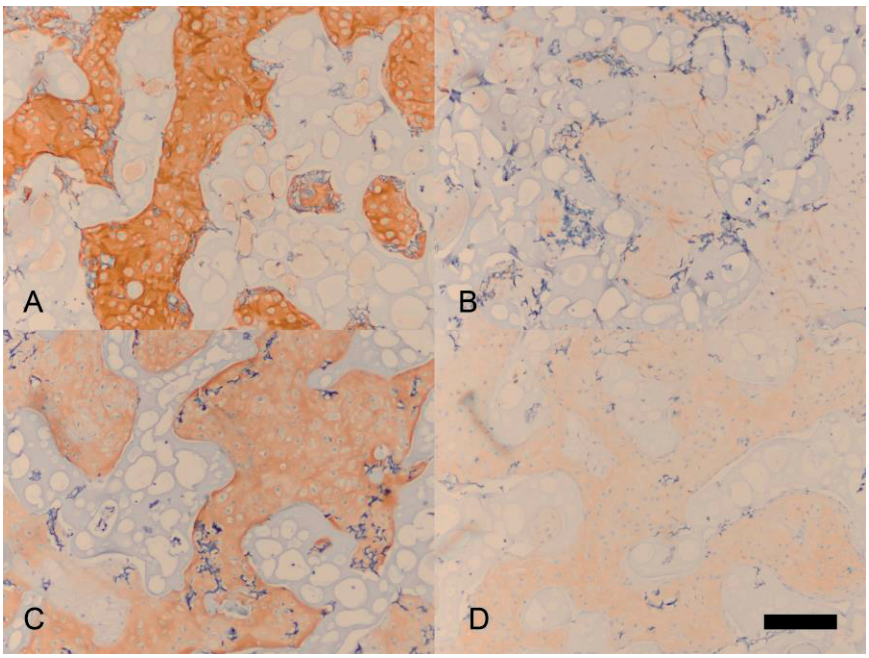

Figure 3. Collagen type II (A, C) and type I (B, D) staining patterns. (A, B) An intense stain for collagen II from the group of PVA-H constructs seeded with articular chondrocytes suspended in FG. A substantially less intense stain for collagen II was found in the group of constructs seeded with chondrocytes suspended in saline (C, D). Both group of constructs demonstrated a less intense collagen I stain than collagen II. The unstained elements represent the porous PVA-H scaffold matrix. Scale bar: 200 mm.

Three-layered construct model. All of the three-layered constructs were recovered and no signs of extrusion or inflammatory reactions, as previously mentioned, were noted at the time of explantation.

The histological and immunohistochemical patterns of stain intensity were similar when compared to the same groups from the disc model, including the presence of neocartilage in group C. Group A was characterized by a lack of integration, identified by a gap between structures (Fig. 4A). In group B, the FG alone did not demonstrate an integrative pattern between the PVA-H and the devitalized cartilage (Fig. 4B). However, group C was characterized by a seamless contiguous integration between structures, demonstrating that the addition of cells assisted in the adherence of the PVA-H to devitalized native articular cartilage (Fig. 4D). The devitalized cartilage from group D also demonstrated integration with the PVA. However, it was intermittent when compared to group C (Fig. 4C).

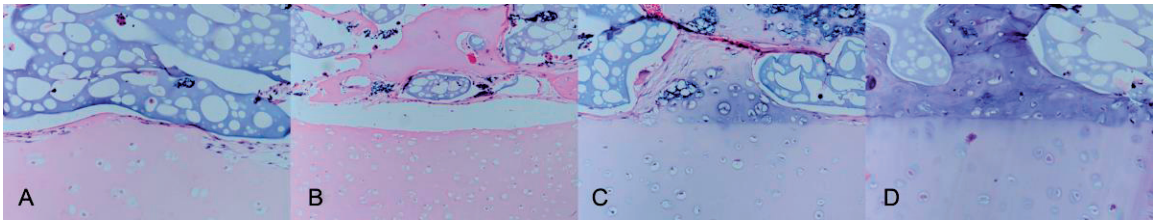

Figure 4. Representative histological sections from the three-layered constructs. (A) PVA+saline, (B) PVA+FG, (C) PVA+saline+cells, and (D) PVA+FG+cells. Acellular constructs failed to demonstrate integration to the porous PVA hydrogel. Notice the gap present in A and B. The addition of cells suspended in saline allowed for partial integration (C), whereas the combining chondrocytes and FG resulted in a continuous interface bonding (D).

Creep testing

The response to creep deformation and subsequent relaxation for both the engineered PVA-H constructs and native swine articular cartilage are shown in Fig. 5 and listed in Table 1. Groups A, B, and D were characterized by a similar response to deformation, demonstrating a total strain of ~ 90% during the first 10 h under 0.5 MPa compressive stress, with a minimal recovery during the subsequent relaxation period under 0.05 MPa compressive stress. Group C demonstrated a TCS of 71%, whereas native swine articular cartilage demonstrated a TCS of 59%. Both group C and native swine cartilage demonstrated a similar elastic, viscoelastic, and strain recovery during the relaxation period. Among the engineered constructs, group C performed most similarly to native swine cartilage (Fig. 5; light green and dark blue lines, respectively).

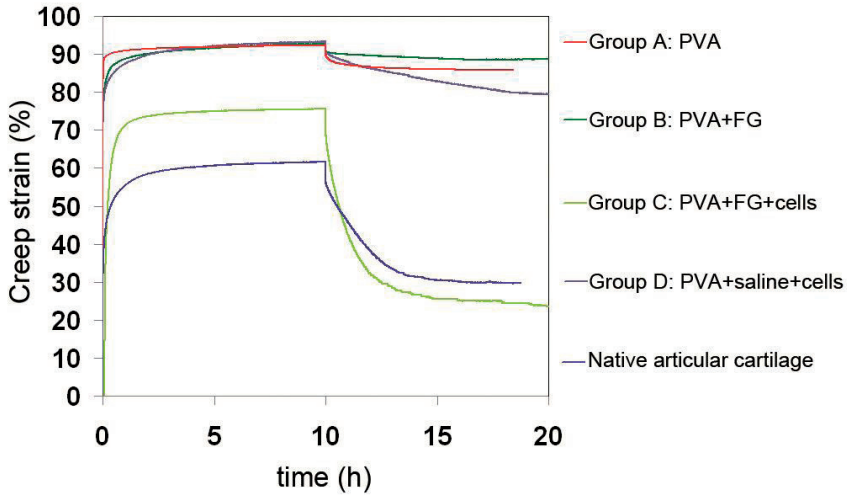

Figure 5. Creep strain (%) over a period of 20 h. The construct containing PVA, FG, and cells performed similarly to native swine articular cartilage. After a 10 h period under 0.5 MPa, both native swine cartilage and group C containing chondrocytes and FG demonstrated a similar viscoelastic response during the recovery period.

Table 1. Total creep strain from the engineered constructs and native articular cartilage

Group	*Contents*	*Total creep strain (%)*
A	PVA	90 ± 5
B	PVA + FG	86 ± 2.5
C	PVA + FG + cells	71 ± 3
D	PVA + saline + cells	88 ± 4
Control	Native cartilage	59 ± 2

FG, fibrin gel; PVA, poly (vinyl alcohol).

Integration strength

The ultimate tensile strength (σ_{UTS}), energy to failure (E_f), Young's modulus ($E\gamma$), and the failure strain (ε_f) measured in the three-layered constructs are summarized in Fig. 6. The presence of cells and fibrin glue significantly enhanced the integration strength of layered constructs ($p<0.05$ for both parameters by two-way analysis of variance). The combination of cells and fibrin glue increased integration strength more than 11-fold from 12 kPa for group B to 133 kPa for group C. The ultimate tensile strength and energy to failure for group C were significantly higher than those measured with groups B and D. The σ_{UTS} and E_f for group D were significantly higher than that for group A. The Young's modulus for group C was significantly higher than that measured with groups B and D. The fracture strain was significantly higher in group B versus group A; and that was also significantly higher in group D versus group A.

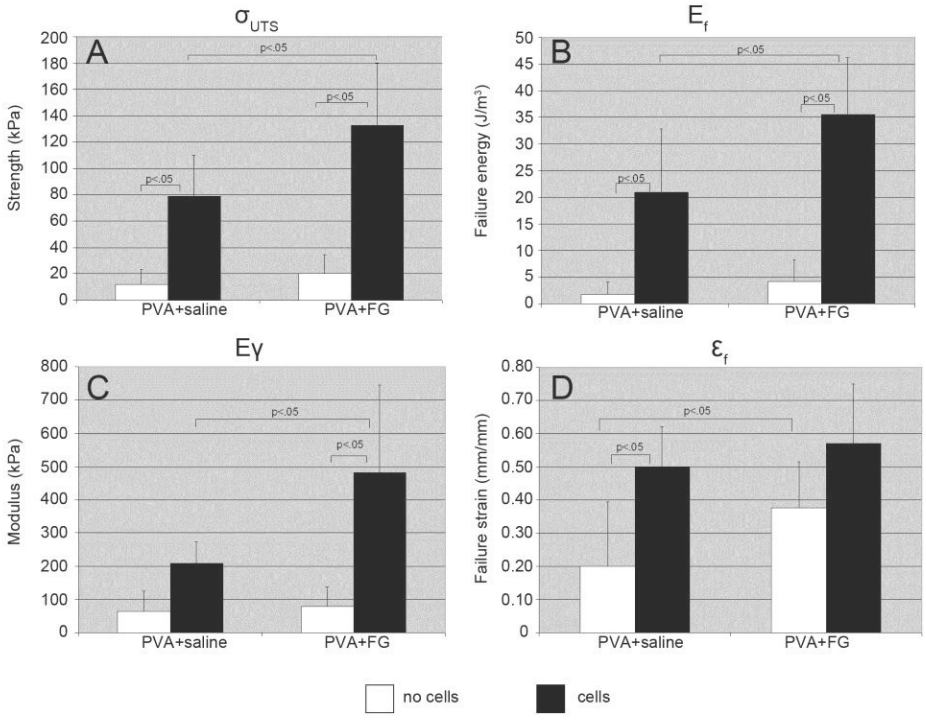

Figure 6. Integration strength testing. Ultimate tensile strength (A), energy to failure (B), Young's modulus (C), and failure strain (D). The presence of cells and fibrin glue significantly enhanced the integration strength of layered constructs.

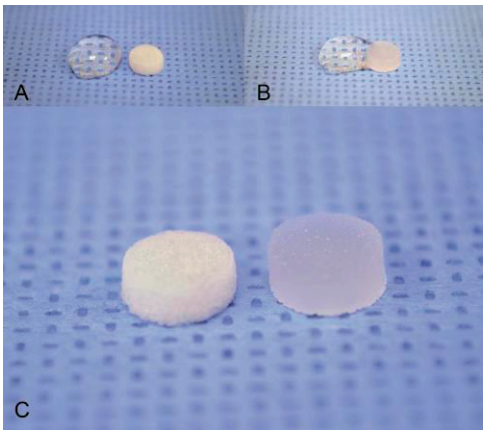

Figure 7. Porous nature of synthesized PVA-H. (A, B) A seamless and homogeneous solution distribution throughout the hydrogel can be visualized. A representative example of actual constructs before (C, left) and after (C, right) cell seeding is pictured. Construct dimensions: 6 mm diameter and 2.1 mm height.

DISCUSSION

The general aim of this study was to create a hybrid tissue-engineered structure with a non-resorbable porous scaffold whereby the open channels are filled with hyaline cartilage tissue. The results demonstrated that the synthesized highly porous non-degradable PVA-H in conjunction with chondrocytes suspended in fibrin glue located throughout the channels enabled the engineering of a biosynthetic construct that exhibited the histological, immunohistochemical, and biomechanical characteristics of native articular cartilage with good adhesion to devitalized articular cartilage. The electron microscopy imaging of the scaffolds revealed a highly porous architecture with an interconnected channel structure that proved ideal for the seeding of chondrocytes (Fig. 1A). There were also some spherical cavities that appeared to be trapped within the matrix, where we did not expect any neocartilage formation. We were not expecting cell attachment to the internal PVA-H surfaces; rather, we were interested in creating ECM with the seeded chondrocytes so as to embed and suspend these cells within the open channels once the FG degraded. The histology primarily showed the cells to be embedded within the ECM.

We initially investigated in a number of *in vitro* preliminary studies an approach that would result in a homogeneous cell infiltration throughout the porous PVA-H described in this study. Our first attempt to seed chondrocytes involved pipetting cells suspended in saline on the top surface of the PVA-H. Not surprisingly, the solution was fully absorbed by the hydrogel (Fig. 7). However, this resulted in a suboptimal cell infiltration throughout the thickness of the scaffold. We then proceeded to seed cells by injection using a 25-gauge needle inserted in the middle part of the scaffolds. The force exerted on the syringe allowed the cells to homogeneously distribute across the channels. However, the solution easily seeped out the PVAH upon minimal manipulation, due in part to the highly porous architecture. This led to the use fibrin glue, which allowed cell encapsulation and prevented subsequent cell loss during handling and implantation of the scaffolds in nude mice.

Engineering articular neocartilage using FG has been extensively studied in the past by our group[57, 60, 76, 79]. By mixing equal volumes of fibrinogen and thrombin, FG can fill irregularly shaped cartilage defects, and as chondrocytes deposit ECM, neocartilage can form and integrate with native tissues[194]. Compared to native articular cartilage, FG is a mechanically weak hydrogel that would be unable to withstand the forces that occur during ambulation. In this study, the PVA-H matrix provided an additional structural support before the chondrocytes that were suspended in FG deposited ECM and improved the mechanical properties of the construct. The mechanical properties

of the PVA-H matrix significantly improved with the addition of cells and FG carrier. We also decided to include saline as a control cell carrier since it would not provide any additional structural support to the PVA gel, unlike FG. This allowed us to compare two groups containing chondrocytes suspended in two different carriers.

The chondrocytes seeded into the PVA-H matrix demonstrated, through histological and immunohistochemical findings, contiguous neocartilage formation after being implanted *in vivo*. The difference between the staining patterns among groups is likely due to the number of cells that remained in the PVA-H that used saline as a carrier between the time of construct creation and ECM production. Preliminary studies, as previously mentioned, revealed seepage of cell solution out of the PVA-H when only saline was used. On the other spectrum, FG acted as a second matrix, preventing chondrocyte-loss through the highly porous structure. To address this issue, various copolymer blends including PVA-poly(lactic/glycolic acid) and PVA-PEG have been studied to enhance and aid in cell adhesion and migration, enhancing tissue formation[195-197]. However, the use of hydrogels including fibrin can equally address this issue, as demonstrated in this and previous studies[191].

Although PVA hydrogels are ideal biomaterials that can be formulated to resemble and replace arthritic cartilage, lack of PVA integration with native tissue has been shown to be a challenge[198]. When attempting to repair focal cartilage defects, a deficient or intermittent integration to host tissue will inevitably lead to construct displacement unless it is mechanically secured. In this study we demonstrated adhesion of inert PVA-H, through the addition of chondrocytes and FG, to native devitalized articular cartilage. The results from the mechanical testing suggest that the biosynthetic construct can mimic the properties of native cartilage and that it can integrate with devitalized tissue. In regard to the creep response, creep strain applied over a period of 20 h resulted in the construct containing FG and chondrocytes (group C) behaving most similarly to native cartilage. The response for both native cartilage and group C during the relaxation period demonstrated similar elastic recovery curves upon unloading, as well as a viscoelastic strain recovery. These findings correlated with the integration strength testing where the combination of cells and fibrin glue significantly increased the integration strength of the structures. The lack of integration of the PVA-H that was injected with acellular fibrin glue with the devitalized cartilage indicated that presence of the cells was necessary to ensure adherence of the PVA-H scaffold to cartilage. These findings are encouraging and the preclinical application in large animal models should be considered. However, other injectable carriers, including alginate, PEG, and collagen hydrogels, should be additionally investigated in case an unfavorable reaction to the bovine-base FG carrier is encountered in an *in vivo* immunocompetent environment.

CONCLUSIONS

Multiple PVA-H formulations have been engineered to mimic the properties of native articular cartilage. In the present study we combined a porous PVA in conjunction with FG and articular chondrocytes to create a hydrogel matrix-engineered biosynthetic cartilage construct. Implantation into immunocompetent animal models and long-term integration are critical factors that must be considered for the successful repair and regeneration of cartilage tissue, as well as the wear characteristics of biosynthetic constructs.

ACKNOWLEGEMENTS

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Chapter 8

Articular cartilage regeneration applying PEG-LA-DM/PEGDM copolymer hydrogels

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ABSTRACT

Injuries to the human native cartilage tissue are particularly problematic because cartilage has little to no ability to heal or regenerate itself. Tissue engineering strategy combining suitable cell sources and biomimetic hydrogels could be a promising alternative to achieve cartilage regeneration. However, the weak mechanical properties may be the major drawback to use fully degradable hydrogels. Besides, most of the fully degradable hydrogels degrade too fast to permit enough extracellular matrix (ECM) production for neocartilage formation. In this study, we demonstrated the feasibility of neocartilage regeneration using swine articular chondrocytes photoencapsulated into poly (ethylene glycol) dimethacrylate (PEGDM) copolymer hydrogels composed of different degradation profile: degradable (PEG-LA-DM) and nondegradable (PEGDM) macromers in molar ratios of 60/40 and 70/30. Articular chondrocytes from juvenile swine were isolated and placed in culture for expansion. The cells were combined with the macromer/photoinitiator (I2959) solution at a concentration of 60 X 10^6 cells/mL. Aliquots of 100 μL of nonpolymerized gel containing the cells were placed in cylindrical molds measuring 4.5 mm diameter X 6.5 mm in height. The gels were polymerized by irradiation of the macromer/photoinitiator/ chondrocyte solutions using ultraviolet (365 nm) light at 10 mW/cm^2 for 10 mins. Also, an articular cartilaginous ring model was used to examine the ability of integration between the engineered cartilage and the adjacent native cartilage. All the samples in experimental group were implanted into nude mice subcutaneously and harvested at 6, 12 and 18 weeks. The non-implanted cylindrical constructs were used as control. All of the harvested specimens were examined grossly and analyzed histologically and biochemically. Histologically, the neocartilage resembled the native articular cartilage in terms of cell configuration and composition of the ECM. Biochemically, there was an increase in the contents of total DNA, glycosaminoglycan and hydroxyproline over the time periods (18 weeks). Integration of neocartilage with existing native cartilage improved with time, resulting in the development of tight integration interface. Articular cartilage regeneration could be achieved using swine articular chondrocytes photoencapsulated into PEGDM copolymer hydrogels, and the neocartilage tissue owned the ability of integration with existing adjacent native cartilage.

INTRODUCTION

Acute destruction of the joint surface or chronic degeneration resulting from the initial trauma can lead to pain and stiffness and loss of joint function. These joint injuries not only limit physical activity and mobility of those afflicted, but the inability to move freely can cause deep psychological scars and loss of independence when individuals have to depend on family and healthcare providers for constant assistance to perform daily life functions. The level of functional capability in the injured limb and ultimate quality of life depend on the successful outcome of joint surface regeneration performed as a secondary procedure weeks or even months after the initial injury. The return of function and the probability of return to active duty rely on successful restoration of the entire joint including the articular surface, and therefore, joint function. To date, however, the outcomes of many restorative procedures are very unsatisfactory and an improved method for joint repair is a clear unmet need in orthopaedic surgery.

Lesions in the joint surface are commonly treated with microfracture[10], autologous cell implantation (ACI)[11], or osteoarticular autograft transfer system (OATS)[12]. Although patients have symptomatic relief, there is no convincing histological or biochemical data to support the contention that the new tissue that forms is characteristic of normal hyaline cartilage found on the joint surface that is comprised predominantly of type II collagen. ACI and microfracture most often result a fibrous cartilage repair that is high in type I collagen and not durable in weightbearing positions over the long-term. Roberts *et al*. have reported that as many as 65 percent of second look biopsies showed fibrocartilage[19]. More modifications of the ACI technique have been tested in Europe involve an open weave or spongeous matrix (MACI), frequently made from collagen or hyaluronic acid, where the cells are absorbed into the matrix before being secured in the lesion[20]. These woven type scaffolds do not provide any immediate biomechanical integrity and can be crushed by the forces placed on the joint. Furthermore, more results suggest that the new tissue formed is also fibrocartilage and the fate of the cells is unknown[21, 22]. The long-term results using MACI techniques are not yet available.

Tissue engineering strategies combining chondrocytes or chondrocyte progenitor cells with biomimetic scaffolds made of natural or synthetic biomaterials could be a promising alternative for cartilage repair and regeneration. Open fibrous scaffolds such as collagen or polyesters have been successful in promoting cartilage formation in immunocompromised animals, but having an open lattice network also permits invasion of inflammatory cells that can negatively affect matrix formation in immune competent animal models[53]. Hydrogels are polymeric materials distinguished by high water content and diverse physical properties. They can be engineered to resemble the extracellular environment of the body's tissues in ways that enable their use in medical implants, biosensors, and drug-delivery devices[51]. Various research groups including ours have successfully demonstrated the neocartilage tissue formation using multiple kinds of hydrogels to encapsulate chondrocytes or mesenchymal stem cells[53, 75, 199-202].

Poly(ethylene) glycol (PEG) is a linear carbon polymer that can be photochemically crosslinked into a stable hydrogel in which the biochemical and biophysical properties can be custom designed to obtain desirable properties for neocartilage formation and permit controlled cartilage matrix production. In collaboration with Drs. Elisseeff and Anseth, we designed a photocrosslinkable hydrogel for cell encapsulation based on PEG[203, 204]. PEG was chosen for the macromer backbone because of its long history in medical applications and desirable chemistry, which allowed easy modification. The PEG polymer chain can be methacrylated, referred to as PEG diacrylate (PEGDM), and in the presence of a photoinitiator (Igracure) and ultraviolet light (365 nm), the polymer forms crosslinks between the linear chains and forms a hydrogel. The polymers and gelation process were designed to provide easy placement and to provide mechanical and structural stability with desirable transport properties during the regeneration process[171]. Work by our collaborator Dr. Anseth at the University of Colorado has shown that both the amount and distribution of extracellular matrix (ECM) by chondrocytes encapsulated in photopolymerized PEG hydrogels *in vitro* is directly correlated to the pore size, degradation rate, and swelling behavior of the gel networks[171, 197, 205, 206]. When the

network mesh pore size is too small (low swelling), the distribution of large ECM molecules (e.g. glycsosaminoglycans) is confined to the pericellular region. By increasing the pore size, the GAG molecules can diffuse throughout the intercellular spaces[205]. Additionally, the degradation properties of the PEG hydrogels (controlled by cross-linking density) can also affect the distribution of ECM. When chondrocytes are encapsulated in nondegradable PEG hydrogels, the collagen molecules are confined to the pericellular area, whereas the collagen is dispersed evenly into the void volume between the cells in gels that are designed to biodegrade[206]. The degradation characteristics can be tailored to make gels that degrade rapidly or slowly over time depending on the amount of degradable lactide units grafted to the PEG.

Several preliminary studies were performed in our laboratory investigating a variety of candidate experimental protocols to develop a novel PEGDM hydrogel that supported chondrocyte survival, growth, and matrix production. The basis of this study derived from our earlier work pertaining to the slow degradation kinetics of copolymer gels and the subsequent extracellular matrix (ECM)-producing potential of chondrocytes in the tissue-engineered cartilage[207].For this study, copolymers composed of degradable poly(ethylene glycol)-4,5 lactic acid dimethacrylate (PEG-LA-DM) and nondegradable PEGDM macromers in molar ratios that ranged from 90/10 to 50/50 were studied. Construct volume and histology (Fig. 1 and Fig. 2) of the engineered cartilage was investigated after 6 weeks *in vivo*, and concluded that the ratios of 60/40 and 70/30 degradable/ nondegradable PEGDM macromers proved more optimal in maintaining the initial construct volume and supporting the formation of neotissue with improved histological features.

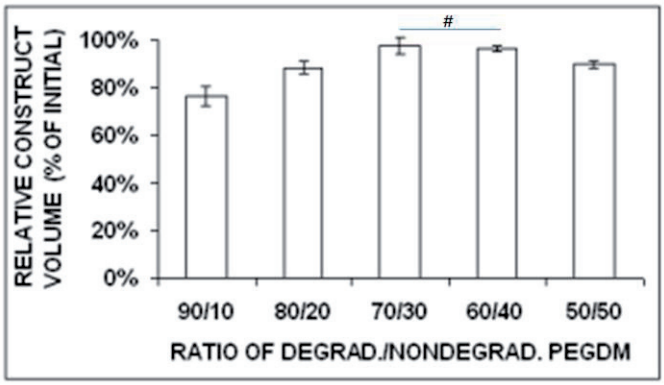

Figure 1. Construct volume data of the preliminary study (# $p>0.05$).

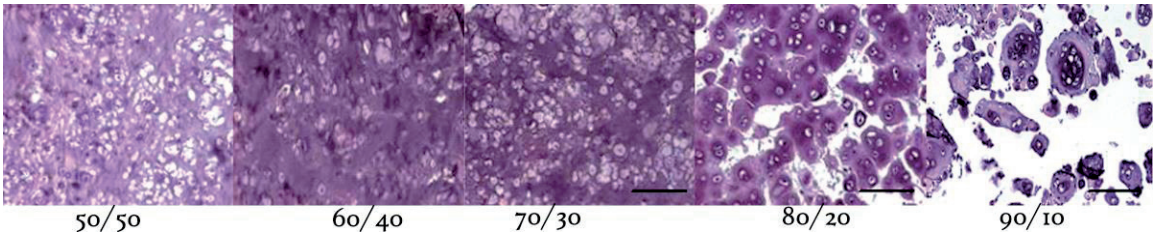

Figure 2. Hematoxylin and eosin-stained sections of the preliminary results demonstrated noncontiguous cartilage formation using 50/50, 60/40, 70/30, 80/20 and 90/10 ratios. (From left to right, original magnification x100, bar: 100 μm)

The aims of our study were: 1 to demonstrate whether neocartilage formation can be achieved using articular chondrocytes photoencapsulated into the combination of degradable and nondegradable PEGDM copolymer hydrogels; and 2 to investigate if the neocartilage can integrate with the existing adjacent native cartilage.

MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital.

Chondrocyte isolation

Articular swine cartilage was harvested from knee joints of three- to six- month old Yorkshire pigs and minced into 1 mm^3 pieces. The cartilage chips were digested using 0.05% collagenase type 2 (Worthington Biochemical Co., Freehold, NJ) solution for 16 hours at 37 ^{0}C. After digestion the chondrocyte suspension was filtered through a 100 μm sterile cell strainer (Becton Dickinson, Franklin Lakes, NJ) to remove undigested debris. Cells were washed, centrifuged and suspended in phosphate-buffered saline. Cell number and viability were examined using a hemocytometer and trypan blue dye method, and only cell isolations with viability above 90 percent were used for the further experiments.

Polymer preparation

The synthesis and *in vitro* data have been previously described[207]. Briefly, PEG-LA-DM, a degradable PEG macromer, and PEGDM, a nondegradable form of PEG, were used in the polymer preparation. These macromers were dissolved in sterile phosphate-buffered saline to a final concentration of 10% (w/w) and mixed at molar ratios of 60/40 and 70/30 (PEG-LA-DM:PEGDM). The ultraviolet potoinitiator, 2-hydroxy-1[4-(hydroxyethoxy)phenyl]-2-methyl-1- propanone (I2959), shown to be cytocompatible, was added to the co-macromer solution at a final concentration of 0.05% (w/w).

Chondrocyte photoencapsulation

The isolated chondrocytes were combined with the macromer/photoinitiator solution at a concentration of 60 X 10^6 cells/mL. Aliquots of 100 μL of nonpolymerized gel containing the cells were placed in cylindrical molds measuring 4.5 mm diameter X 6.5 mm in height. The gels were polymerized by irradiation of the macromer/photoinitiator/ chondrocyte solutions using ultraviolet (365 nm) light at ~10 mW/cm^2 for 10 mins. Thirty two specimens were prepared for each polymer mixture.

Ring model preparation

Cartilaginous rings were used to study the integration of the neotissue with existing cartilage matrix[208]. Disks of swine articular cartilage measuring 8 mm X 2mm were made using an 8 mm punch biopsy. The center of the cartilage disk was removed using a 5mm biopsy punch leaving a ring of native matrix. The cartilage rings were devitalized using five freeze–thaw cycles. The central cavities of the disks were filled with the gel containing cells and photopolymerized as described above.

Implantation and harvest

Experimental constructs were implanted subcutaneously into 5 week old athymic male mice (nu/nu) (MGH, Boston, MA). Eight specimens from each group were harvested at 6, 12 and 18 weeks after implantation. Samples were evaluated macroscopically, weighed and randomly selected for either histologic examination or biochemical analyses including total DNA, glycosaminoglycan (GAG) and hydroxyproline analyses. Additional samples (n=8) were prepared for each polymer mixture and used for assays at time zero. The ring model constructs were grossly examined and processed for histology.

Specimen evaluation

At the time of harvest, the wet weight and overall dimensions of the samples were recorded. The specimens were cut in half with one-half submitted for histological processing and the remaining half used for biochemical assays. For the histological processing, the samples were fixed in 10% buffered formalin for 24 h and embedded in paraffin. Serial 5 mm sections were obtained, deparaffinized, and stained with hematoxylin and eosin (H&E) and safranin O to evaluate tissue morphology and proteoglycan content. Additional sections were immunostained for collagen type I and II (Chondrex,

Redmond WA). Briefly, slides were treated with 2% bovine testicular hyaluronidase at room temperature for 30min, followed by a blocking reagent consisting of 0.3% hydrogen peroxide in methanol for another 30 min. Then, 10% goat serum was added to each slide for 30min; on separate slides, antibodies for collagen type I and II were applied for 1 h. For negative control, N-Universal Negative Control was applied, and the secondary antibody was added for 20min. Then, 3,3-diaminobenzidine was applied to each slide, and cell nuclei were counterstained with hematoxylin.

The experimental specimens as well as native articular swine cartilage for biochemical evaluations were weighed to obtain the wet weight of the samples. The samples were lyophilized and weighed again to obtain the dry weight of the specimens. The difference in wet and dry weights gave the water content of the neotissue. The lyophilized specimens were enzymatically digested in papain type III solution (Sigma-Aldrich, St. Louis, MO) at 125 mg/mL at 60^{o}C for 16–24 h. DNA content was determined using the PicoGreen dsDNA Quantitation Assay Kit (Molecular Probes, Eugene, Oregon). A five-point standard curve of Lambda DNA was plotted. The amount of DNA content was determined from the curve and used as indicator of the proliferative potential of the photoencapsulated chondrocytes. GAG content of the constructs was measured using the dimethylmethylene blue dye method[209]. Chondroitin sulfate B (Sigma-Aldrich) was used as a standard in the interpretation of the data. The hydroxyproline content was quantified using the simplified hydroxyproline method as previously reported[210]. L-4- Hydroxyproline (Fluka Biochemika, Steinleim, Switzerland) as used as standard in a seven point curve. All the biochemical values were normalized by wet tissue weight.

Statistical analysis

Statistical analysis was performed on the quantitative data of the study using a Student t- test (Sigmastat 2.0, SPSS Science, Chicago, IL) and ANOVA. Level of statistical significance was set at 0.05 and all the values are reported as the mean ± standard deviation.

RESULTS

Gross evaluation

A reduction in average wet weight was showed at 6 weeks in both copolymer hydrogel groups compared with the control samples, particularly in the less degradable gel group ($p<0.05$ for 60/40 group and there's no statistically difference for 70/30 group). However, by 18 weeks, the average wet weight of the samples appeared higher than the initial one in both groups ($p<0.01$). A reduction in average volume was showed at 6 weeks in 60/40 copolymer hydrogel group ($p<0.05$), whereas there's no significantly reduction in volume showed in 70/30 copolymer hydrogel group at the same time point. Still, by 18 weeks, the volume of samples appeared higher than the initial one in both groups ($p<0.01$). There's slight reduction in average water content showed at 6 weeks in both copolymer hydrogel groups compared with the control samples, and by 18 weeks, there's slight increase in average water content showed in both groups. However, there's no statistically different among the study groups or harvest time points. (Table 1)

Table 1. All the data are presented as mean value ± standard deviation. Data of DNA, glycosaminoglycan (GAG) and hydroxyproline (HYP) assays were normalized by the wet weight of samples. DNA content is shown as percentage of the initial one (that of control, non-implanted samples)

	60/40 (PEG-LA-DM/PEGDM)				70/30 (PEG-LA-DM/PEGDM)			
	Control	6w	12w	18w	Control	6w	12w	18w
Wet weight (mg)	103.50±4.10	91.35±4.31	111.05±4.59	117.90±4.38	104.80±8.06	100.35±3.61	107.45±2.57	114.30±2.54
Volume (mm3)	93.54±1.21	86.09±3.92	96.02±3.89	104.55±1.32	92.92±4.64	91.87±1.42	93.79±1.38	102.85±2.37
Water content (%)	82.29±3.78	79.01±4.05	85.70±2.45	86.09±2.95	83.14±3.87	80.07±4.42	86.22±3.17	85.61±2.91
DNA (% of initial)	100	137.76±19.43	173.97±19.56	184.29±29.19	100	160.73±20.55	184.42±30.91	206.74±32.62
GAG (µg/mg)	6.86±2.23	21.16±3.89	22.89±3.39	35.32±8.67	7.64±2.03	16.62±5.44	26.64±3.49	33.46±7.32
HYP (µg/mg)	0.82±0.34	2.89±1.14	5.24±0.89	7.61±0.60	0.86±0.54	3.52±1.15	6.59±1.08	9.52±3.01

Macroscopically, the samples became increasingly opaque over time (Fig. 3). While the samples at 6 weeks still presented large areas of translucency due to the gel component, the samples at 18 weeks were completely opaque. The consistency of the samples at 6 weeks was relatively soft and gel-like, but as time increased the gel was replaced with new cartilage matrix and the nodules became stiffer.

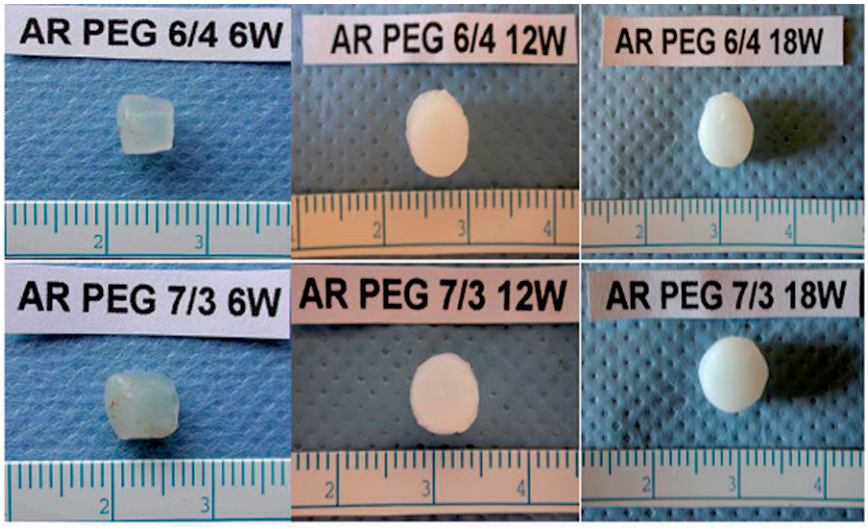

Figure 3. Macroscopic view of constructs over the implantation time.

Biochemical evaluation

DNA content data was evaluated as percentage of the control sample DNA content values measured at time zero that was set at 100% (Fig. 4). In both groups, DNA content was increased throughout the

study. At 18 weeks, the mean value of DNA content was 184.29±29.19 and 206.74±32.62 for both 60/40 and 70/30 hydrogel groups, respectively. No significant difference was found among the study groups at any harvest time point.

The GAG content of the neotissue (Fig. 4) demonstrated a very high increase ($p<0.01$) during the first 6 weeks of implantation in both copolymer hydrogel groups. The amount of GAG content increased with time as well. At 18 weeks, the amount of GAG was 35.32±8.67 µg/mg of wet tissue weight in the 60/40 group, whereas in the 70/30 group it was slightly lower, 33.46±7.32 µmg/mg. No statistically significant difference was observed between different study groups at all harvest time points. On the basis of data from our preliminary biochemical study of swine native articular cartilage, the GAG content of engineered cartilage using both 60/40 and 70/30 ratio of PEGDM hydrogels was 63.39% of that in native cartilage, whereas the GAG content of the neotissue made with 70/30 gel was 60.06% of that in native articular cartilage.

In both groups, the hydroxyproline content (Fig. 4) also increased over time. At the final harvest time point the total collagen was of 7.61±0.60 µg/mg of wet tissue weight in 60/40 hydrogel group, approximating 56.52% of that found in the native swine articular cartilage. The amount of total collagen in the 70:30 hydrogel group was slightly higher, 9.52±3.01 µg/mg or about 70.67% of the content measured in native cartilage. There was no significant difference between groups, although both groups only reached about a half to two-thirds of the total collagen of native articular cartilage in the time frame of this study.

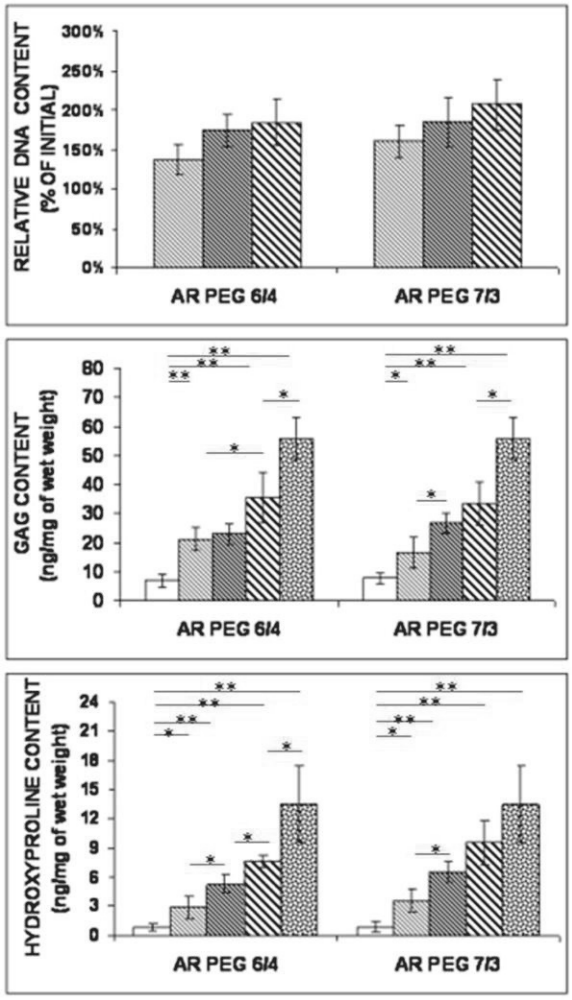

Figure 4. Biochemical evaluation data. (a) DNA content (b) GAG content and (c) hydroxyproline content. (* $p<0.05$, ** $p<0.01$.) □ 0 w, ▨ 6 w, ▨ 12 w, ▨ 18 w, ▨ native cartilage

Histological evaluation

Tissue sections stained with hematoxylin and eosin (Fig. 2) revealed that the chondrocytes and ECM of the neocartilage resembled morphology of that observed in native swine articular cartilage. The presence of dispersed ovoid-shaped chondrocytes located within typical rounded lacunae and surrounded by a basophilic matrix was observed. Nonhomogeneous distribution of the encapsulated chondrocytes was observed in both study groups at 6 weeks, probably due to the incomplete degradation of the hydrolysable crosslinks of the polymer scaffold at that time point. No visual difference was observed between the 60/40 and 70/30 groups.

Immunostaining for collagen type I and II demonstrated that the encapsulated chondrocytes maintained their phenotype and produced collagens typical of that found in native cartilage (Fig. 5). Intense positive staining for collagen type II was observed throughout the ECM of the constructs in both groups. In the 6-week specimens, the type II collagen was mainly confined to the immediate pericellular regions, presumably due to the incomplete degradation of the polymer. In later samples, the type II collagen staining pattern was more homogeneous throughout the specimens.

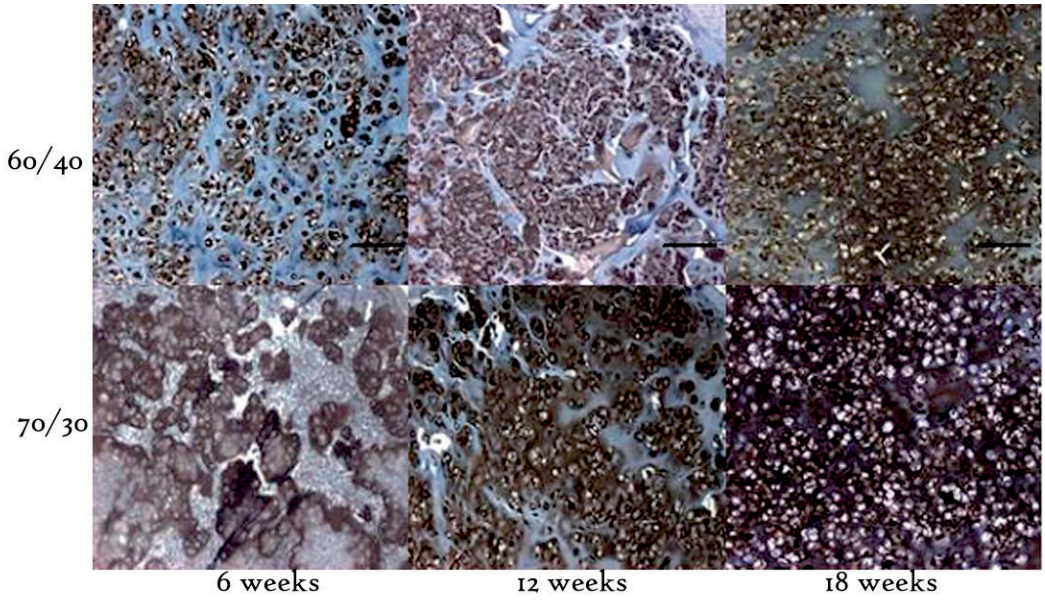

Figure 5. Comparative evaluation of the type II collagen immunostaining results regarding the different experimental copolymer hydrogel groups through the *in vivo* time. (From left to right: 6, 12 and 18 weeks. Upper row: 60/40 gel group, lower row: 70/30 gel group. Original magnification x 40, bar: 100μm)

Results from the cartilage ring constructs demonstrated integration of the neotissue with the existing native cartilage in the ring. The cartilage rings were devitalized to eliminate the possibility of chondrocytes from the ring migrating into the repair interface and influencing the results. In samples at 18 weeks, the integration interface was extensive and involved almost all the surface of the surrounding native cartilage evidenced by Toluidine Blue staining and Type II collagen staining (Fig. 6). Attaching cells presented ovoid in shape and most often aligned perpendicular to the surface of the native cartilage. The engineered tissue appeared to closely follow the configuration of the devitalized cartilage surface and to fill irregularities of various depth and shape along the integration interface. No obvious morphological differences in the integration pattern were observed between the two copolymer groups studied.

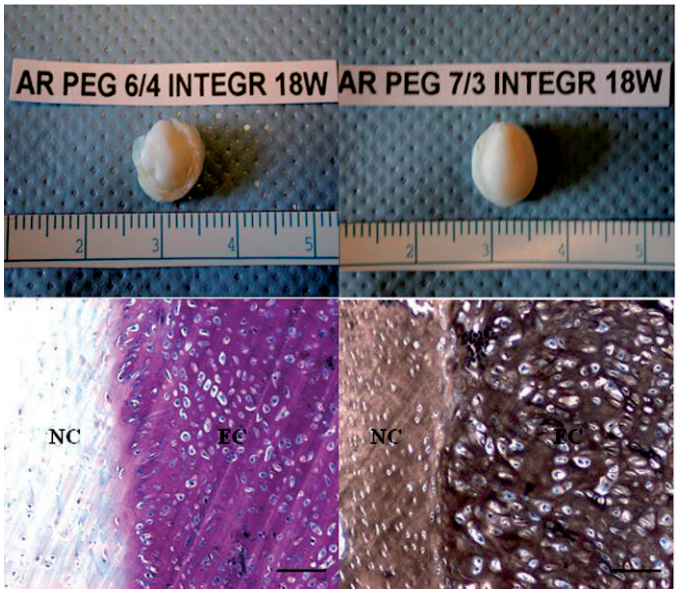

Figure 6. Upper row: Macroscopic view of 18 w constructs of ring model for integration study. Lower row: Histological staining of the integration interface between the engineered cartilage (EC) and native articular cartilage (NC) related to the above presented constructs. Toluidine Blue O (left) and immunostaining (right) for collagen type II. (Original magnification x 100, bar: 100μm)

DISCUSSION

Development of an effective and minimally invasive tissue engineering technique using injectable biomaterials to support chondrocyte growth and ECM production would be an ideal therapeutic strategy for cartilage repair and regeneration. Hydrogels are water swollen networks, suitable for the delivery of cells and bioactive agents. They may be used as injectable scaffolds since they easily fill defects of any size and shape and may be implanted in a minimally invasive manner. Hydrogels support the transport of nutrients and waste, and can homogenously suspend cells in a three-dimensional environment, where encapsulated cells typically retain a rounded morphology that may induce a chondrocytic phenotype. Hydrogels are also capable of transducing mechanical loads to exert controlled forces on encapsulated cells, similar to physiological conditions. Though their mechanical properties can be altered by crosslinking density (which may compromise cell viability) limited mechanics may be the major drawback to using hydrogels[182]. Besides, most of the degradable hydrogels degrade too fast to permit enough ECM production for neocartilage formation. Nondegradable gels can be formulated that are resistant to erosion and maintain three-dimensional architecture, but do not permit cell-to-cell contact and interfere with ECM production. Degradable gels can be tailored to break down within predictable periods of time. Delicate timing of this process is necessary, however, to be able to maintain volume and three-dimensional shape. Employing photochemical crosslinking techniques to polymerize the gels could allow molding the gels into predetermined shapes and permit cartilage formation in the desired form[53]. Extensive study of fully degradable PEG-LA-DM was not performed because previously published *in vitro* data showed that the scaffold degraded too rapidly for cartilage matrix to form and pilot *in vivo* studies confirmed this as well. Although previous data have shown that fully nondegradable PEGDM gels permit pericellular matrix formation, the resilience of the nondegradable polymer inhibits contiguous cartilage matrix formation. By combining the degradable with the nondegradable formulations, we hoped to exploit the advantages of both types of gels for forming articular cartilage. The purpose of this study was to evaluate the neocartilage formation applying copolymers composed of a combination of degradable and nondegradable PEG macromers.

Our data showed that the average wet weight and average volume of samples appeared statistically higher than the initial one in both groups at 18 weeks after implantation. Macroscopically, the samples

became increasingly opaque over time indicating more ECM deposition. Biochemical changes paralleled the morphological observations over time. There was a significant increase in GAG, hydroxyproline and total DNA content during the 18 weeks' period, and there's no statistically difference between 60/40 and 70/30 groups at each time point. The phenotype of the chondrocytes was preserved as evidenced by increased immunohistochemical staining of type II collagen over time. These findings are not quite consistent with our previous *in vivo* data using swine auricular chondrocytes encapsulated into the same PEG macromers[53]. They are probably related to the chondrocyte sources and are supported by the conclusions of previous study conducted by Xu *et al*., which investigated the effect of various chondrocyte sources on the construct mass and volume[62]. Isogai *et al*. also demonstrated that chondrocytes obtained from different cartilaginous sites in an animal may elicit distinct responses during their respective development of a tissue-engineered neocartilage[211]. Normally, the articular chondrocytes receive nutrition by diffusion from the surrounding synovial fluid, facilitated by the loading patterns of a joint. Ectopic implantation of these cells into subcutaneous space alters dramatically their biological environment, while the nutritive elements are received from the interstitial fluid and the biomechanical stimuli are quite different. Hence, this study also provides valuable information on choosing different chondrocyte sources for different clinical applications.

Integration of the engineered cartilage with existing adjacent cartilage is of great importance in articular cartilage regeneration. A weak interface could cause collapse of the tissue and jeopardize the restored functions This process is appeared to be dependent on viable cells at the interface of integration[212, 213].Integration of neocartilage with recipient tissue appears to be dependent on viable cells at the interface of integration, even in only one of the[213, 214].The adhesive strength of cartilage integration has been correlated with collagen synthesis and deposition[215]. Investigating the integrative properties of the PEGDM-based neocartilage, we found that the engineered hydrogels can support the integration of the engineered cartilage with existing native cartilage. At the early 6-week time point the samples demonstrated only partial integration with the opposing surface of the native cartilage, but by week 12 and 18, an improvement of the integrative properties of the engineered cartilage was observed. The integration interface exhibited evidence of repopulation, deposition of matrix macromolecules, and tight adhesion of the engineered cartilage. The breakdown of the polymer scaffold over time could have influenced the properties of the neotissue, leading to improved integrative properties and a tight interface junction at the later times.

Most studies evaluating polymer scaffolds seeded with cells have been pilot tested by implantation in nude mice, thus the refinement of materials for generating cartilage and bone can be done in athymic (nude) mice. Nude mice lack a repertoire of mature T-cells and cannot mount a cellular rejection response to allogeneic or xenogeneic cells. In many ways they can be considered a "living" petri dish for studying cartilage (and bone) matrix formation from cell-seeded scaffolds. We chose a mouse model with subcutaneous implantation to generate neocartilage because this permits better cartilage formation than *in vitro* systems. The subcutaneous pockets on the back of nude mice do not, however, replicate the biomechanical microenvironment in the joint. We agree that the model may have some deficiencies, but the nude mouse is a suitable small animal model to pilot screen and test new scaffolds and tissue formation. Further modifications in hydrogel chemistry or photopolymerization mechanism can improve the scaffold properties of PEG derivatives for tissue engineering of articular cartilage, particularly in terms of the intrinsic adhesive properties. The application of these copolymers in immunocompetent animal models will also be our next focus in this study.

CONCLUSIONS

Our findings demonstrated that copolymers that are composed of degradable and nondegradable PEGDM macromers can serve as scaffold in tissue engineering of articular cartilage. No obvious differences were observed among the studied copolymers at different ratios (60/40 and 70/30) in the cell proliferation, biochemical performance and integrative properties between the engineered tissue and adjacent native tissue. Potentially, a liquid macromer/chondrocyte suspension like PEGDM

copolymer could be injected into the cartilaginous defect and photopolymerized to provide a minimally invasive technique to promote or enhance articular cartilage repair. Further studies are required to investigate the potential of this tissue engineering approach for chondrogenesis involving large animal models and focusing on the biomechanical properties of the engineered articular cartilage.

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Chapter 9

Discussion, conclusions and future perspectives

Healing native cartilage is a significant clinical challenge since the tissue has little capacity to self-regenerate[216]. Tissue engineering strategies utilizing the cell-seeded scaffolds to generate viable, implantable tissues, which can then replace, restore, and/or maintain function in sites of injury and damage *in vivo*[217], present a promising treatment option for cartilage repair and regeneration. Hence, the suitable cell sources and scaffold materials are two of the vital components for achieving cartilage repair and the regeneration aim. Therefore, the goal of this dissertation is to evaluate different cell sources, including chondrocytes and mesenchymal stem cells (MSCs) grown in chondrocyte-conditioned media, and to explore novel maneuvers of improving biomimetic hydrogels as scaffold materials for cartilage repair and regeneration. In this chapter, the results of the studies described in this dissertation are discussed and interpreted, allowing to draw some conclusions and future perspectives.

The first set of aims of this dissertation was to evaluate different cell sources for the generation of cartilage.

Specific aim 1: To evaluate the feasibility of cartilage generation using middle-aged human chondrocytes seeded in a biomimetic hydrogel (Chapter 2).

Autologous chondrocyte implantation (ACI) represents one of the first tissue engineering applications for the regeneration of the articular cartilage surface and has paved the way for research into therapies that combine cells with scaffold carriers[11, 68, 216]. Carticel® developed by Genzyme was the first and only FDA-approved cell therapy product used to repair articular cartilage in the US[218]. Modifications of ACI involving scaffold-supported ACI have been introduced clinically in Europe for treatment of larger chondral defects (> 2 cm^2). These types of procedures require open surgery and re-implantation of *ex vivo* cultured autologous chondrocytes harvested as a biopsy arthroscopically in a prior surgery. This two-step procedure has provided resolution to thousands of patients and has been shown to have a significantly better and more durable result than natural healing wound repair. However the downside of complex logistic, considerable costs have also shown areas of possible improvement[69, 219-221]. Nonetheless, the concept of using terminally differentiated chondrocytes to generate new cartilage ECM has been established through these clinical studies of ACI.

Numerous research groups have developed methodologies to generate cartilage tissue through cell encapsulation into hydrogels or seeding them onto scaffolds[59-62, 67]. However, the vast majority of these studies have used cells from juvenile animal sources—cells that are believed to have a strong propensity to form new ECM[30]. Although cartilage can be easily generated with chondrocytes from juvenile animals, these favorable results may not be an accurate model for the clinical conditions of patients requiring treatment for articular cartilage lesions, many of whom are middle aged or older, or have joints with disturbed homeostasis thus less healthy chondrocytes to start the culture process with. The primary aim of this study was to engineer neocartilage tissue from 50–60-year-old human chondrocytes and compare the results to engineered cartilage made from juvenile swine chondrocytes. The results from Chapter 2 demonstrated that cartilage ECM formation using articular chondrocytes from middle-aged people can be achieved in a predictable and reliable manner. Although the data obtained using human chondrocytes were not identical to cartilage engineered with juvenile swine chondrocytes, there were many similarities noted in histological appearance, collagen and GAG production, as well as the mechanical performance of the new ECM. Those discoveries make this head-to-head study comparing juvenile swine chondrocytes to human cells from middle-aged people to engineer cartilage more meaningful because it moves these cell–scaffold technologies closer to possible clinical application. To our knowledge, this study was the first direct comparison study between middle-aged/elderly human chondrocytes and juvenile animal chondrocytes using a hydrogel system *in vivo*. In conclusion, the results show that the data gleaned from juvenile animal studies can possibly be translated to clinical application for engineering cartilage.

Specific aim 2: To evaluate the feasibility of: 1) generating auricular cartilage using sheep chondrocytes; and 2) using sheep bone marrow-derived mesenchymal stem cells (BMSCs)

grown in conditioned media collected from chondrocyte cultures and biomimetic scaffolds (Chapter 3-4).

The first goal of these studies was to engineer auricular cartilage tissue using sheep ear chondrocytes seeded on a fibrous collagen scaffold. Whereas repairing acute defects in the joint surface involve filling the defined lesion with cells or scaffold materials, ear reconstruction generally involves the entire external auricle protruding from the side of the cranium. This presents several challenges in growing sufficient amounts of cartilage that can maintain a complex three-dimensional structure in a subcutaneous wound bed. Several prior studies have used hydrogels combined with animal ear cells to generate auricular cartilage. However, gels are mechanically weak and require external stenting to retain the three-dimensional architecture of the ear when placed subcutaneously. For this reason, a fibrous collagen type I/III scaffold was chosen. This material can be manipulated and strengthened to withstand the forces of the overlying skin and could retain the three-dimensional relief of the ear.

The results from this study demonstrated that new ear cartilage matrix could be formed using sheep auricular chondrocytes and this fibrous collagen scaffold *in vivo* in mice. The secondary objective of the study was to explore means to preculture the cell-scaffolds in an attempt to improve their cartilage forming capacity after *in vivo* implantation. Scaffolds seeded with cells and placed in motion showed slightly improved cartilage formation over those in static culture. This may be the result of stimulation of matrix formation prior to implantation in the mice. In conclusion, these conditions permitted uniform cartilage matrix formation. Whereas the availability of autologous chondrocytes for ACI is established, the limited availability of autologous auricular chondrocytes in patients without ear(s) may preclude this strategy of using primary chondrocytes for a tissue engineered reconstruction. Hence, more reliable and practical cell sources should be discovered to achieve cartilage repair and regeneration.

MSC sources could present an excellent opportunity if they can be harvested in sufficient numbers and can be directed down the appropriate chondrogenic pathway. The BMSCs study was the key part of this aim to determine if chondrocyte-conditioned medium collected from cultured auricular chondrocytes could promote chondrogenic differentiation of BMSCs towards auricular cartilage matrix formation. For comparison, we used the standard protocol of using exogenous recombinant TGF-β3 to differentiate the MSCs. In recent years, stem cells have generated significant interest in cartilage tissue engineering as an alternative to autologous chondrocytes[182]. Mesenchymal stem cells are multipotent progenitor cells and have the capacity to differentiate into a variety of connective tissue cells including bone, cartilage, and adipose tissue both *in vitro* and *in vivo*[222]. MSCs can be derived from many tissues including bone marrow, adipose tissue, synovium, and umbilical cord[223-227]. One of the greatest challenges for using MSCs to generate cartilage, however, is directing the cells down the desired chondrogenic differentiation pathway[35]. Although many different growth factors have worked for chondrogenic differentiation with varying results[36, 121], transforming growth factor-beta 3 (TGF-β3) has been shown to be one of the most efficient growth factors for inducing chondrogenic differentiation of stem cells[122, 123]. However, MSCs may exhibit a hypertrophic phenotype under chondrogenic induction resulting in calcification after ectopic transplantation[38, 39]. While exogenous recombinant growth factors have been shown to be useful in laboratory studies, clinical application of growth factors has been shown to be troublesome and creates mixed results and most have not been approved by the U.S. Food and Drug Administration (FDA) for clinical use[37].

Performance of cell sources in cartilage formation can be augmented by making use of co-culture strategies. Co-culture of different cell sources are based on the idea that the multi-signal events *in vivo* cannot be perfectly mimicked by adding a limited variety of growth factors to a monoculture of which the optimal cocktail remains largely elusive. This problem can be circumvented by the introduction of another cell source in the culture. In this way, cells are exposed to a wider variety of stimuli, possibly through autologous nonrecombinant (secreted soluble) factors. Such stimulation by naturally produced soluble factors could overcome the regulatory issues regarding the use of nonautologous recombinant factors in clinical settings[47]. The results presented in Chapter 4 using conditioned-media collected from chondrocytes presents a unique means to induce chondrogenesis of MSCs and avoids the direct contact between different cell sources. Real-time PCR results showed up regulation

of COL2A1 in the constructs cultured in chondrocyte-conditioned medium (CCM) compared to those in MSCs standard medium (SM). After 12 weeks *in vivo*, abundant neo-cartilage formation was observed in the implants that had been cultured in CCM, with or without TGF-β3. In contrast, very little cartilage matrix formation was observed within the SM groups, regardless of the presence of TGF-β3. Osteogenesis was only observed in the SM group with TGF-β3. We concluded that CCM even had a stronger influence on chondrogenesis than the supplementation of the standard culture medium with TGF-β3, without signs of endochondral ossification. Apparently, efficient chondrogenic differentiation of BMSCs by using CCM could provide a promising alternative cell population for cartilage regeneration. To our knowledge, this is one of the few studies using chondrocyte-conditioned medium to successfully generate auricular cartilage tissue from MSCs, and, most importantly, this was demonstrated *in vivo*.

Although conditioned medium/co-culture strategy has shown a great potential for inducing chondrogenesis, more studies focused on the mechanism[228] of chondrogenic induction are necessary for understanding efficient differentiation of MSCs towards chondrogenesis and, subsequently, cartilage matrix formation.

Concise implications of "cell sources" part:

Tissue engineering with chondrocytes or other chondroprogenitor cells is now considered to be a promising technique for repairing and regenerating cartilage[16]. The ideal cell source for cartilage tissue engineering, however, is one that can easily be isolated, expanded and synthesizes abundant cartilage-specific extra-cellular matrix components. Since chondrocytes are the terminally differentiated cells found in native cartilage, they could be considered the most obvious cell source. For example, FDA-approved ACI has been in clinical use since 1987 and has been performed on over 12,000 patients worldwide[17]. Thus, autologous chondrocytes may continue to be the most reliable and realistic cell source for cartilage repair in the near future.

In most cartilage tissue engineering studies chondrocytes from immature animals are used, which are believed to proliferate faster and have increased chondrogenic potential compared to chondrocytes from older human donors[229, 230]. This is crucial since patients sustaining acute joint surface injuries may more likely be middle aged or older. The results from the study presented in Chapter 2 demonstrated that middle-aged human chondrocytes are capable of producing new cartilage ECM that is similar in many ways to tissue-engineered cartilage using juvenile animal chondrocytes. These results form the basis for engineering cartilage with degradable hydrogel scaffolds in this patient population, which will provide valuable experimental proof to apply autologous chondrocytes combined with scaffolds to repair cartilage defect in clinics in the near future. This information will add to the knowledge base already established using ACI and provide a sound basis for moving chondrocyte-scaffold therapies toward clinical application.

Engineering auricular cartilage shares much with generating articular cartilage, but the ear presents other challenges. The work presented in Chapter 3 shows that new auricular cartilage can be formed on a fibrous collagen scaffold that has been subjected to motion in the seeding and preculture phases. However, the availability of primary autologous ear chondrocytes from the patient may limit the application of an engineered cartilage replacement. This is a major obstacle in the clinic, particularly when sufficient numbers of phenotypically competent cells are not at hand. One possible solution for overcoming the limited supply of primary chondrocytes is the use of MSCs, possibly from bone marrow or adipose tissue[231]. The major challenge for using MSCs is directing them down the appropriate chondrogenic pathway in order to obtain the desired tissue type. Large numbers of published *in vitro* studies have demonstrated that MSCs can be differentiated into cartilage-forming lineages using various conditions and exogenous recombinant growth factors. However, there is no consensus on the optimum growth factor and culture conditions needed to drive differentiation of MSCs to a stable chondrocyte phenotype[16]. We sought to use the natural, soluble growth factors produced by chondrocytes to stimulate differentiation and form new cartilage matrix. Our studies demonstrated that chondrocyte-conditioned medium (CCM) was effective in inducing chondrogenic differentiation in BMSCs. Specifically, CCM had a stronger influence on chondrogenesis than supplementation of the standard culture medium with TGF-β3 without inducing calcification, which

provides a novel strategy to enhance chondrognesis of MSCs. The elastin produced by BMSCs grown in CCM makes the specific auricular regeneration more realistic. This type of strategy may avoid some of the regulatory hurdles of using recombinant growth factors and will benefit the application of using stem cells for cartilage repair in the future.

The second goal of this dissertation was to explore the novel techniques to improve biomimetic hydrogels as "cell carriers" for cartilage regeneration.

Specific aim 1: To evaluate the feasibility of cartilage regeneration using photochemically crosslinked hydrogels (Chapter 5-6).

The primary aim of these studies was to engineer neocartilage tissue using swine articular chondrocytes encapsulated into photochemically crosslinked hydrogels. Chapter 5 reports on a dual crosslinking paradigm consisting of (a) photocrosslinking with Rose Bengal (RB) and green light followed by (b) chemical crosslinking with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and N-hydroxysuccinimide (NHS) to enhance type I collagen gel stiffness, while retaining favorable cell viability. Chapter 6 presents data on a photochemically crosslinked gelatin-methacrylamide (GelMA) hydrogel to provide a favorable microenvironment for both articular and auricular chondrocytes allowing them to produce extracellular matrix. The results of this study showed that the engineered neocartilage was similar to native cartilage histologically and biochemically.

Numerous scaffold materials have been used for cell delivery in cartilage regeneration. Scaffolds provide a three-dimensional environment that is desirable for the production of cartilaginous tissue. Ideally the scaffold should: 1) have directed and controlled degradation, 2) promote cell viability, differentiation, and ECM production, 3) allow for the diffusion of nutrients and waste products, 4) adhere and integrate with the surrounding native cartilage, 5) span and assume the size of the defect, and 6) provide mechanical integrity depending on the defect location[182]. Hydrogels are water-swollen networks, suitable for the delivery of cells and bioactive agents. Hydrogels may be used as injectable scaffolds since they easily fill defects of any size and shape and may be implanted in a minimally invasive manner. Hydrogels support the transport of nutrients and waste and can homogenously suspend cells in a three-dimensional environment, where encapsulated cells typically retain a rounded morphology maintaining a chondrocytic phenotype. Hydrogels, especially formed from naturally derived polymers, such as agarose, alginate, chitosan, hyaluronan, collagen, fibrin, and polysaccharides, are attractive biomaterials because they are biochemically similar to cartilage and can be degraded by cell-secreted enzymes[54]. Though their mechanical properties can be altered by crosslinking density (which may compromise cell viability) limited mechanics may be the major drawback to using hydrogels[182]. Therefore, researchers have been trying to tailor the mechanical properties of hydrogels by different means, such as the application of bioprinting[232, 233]. In this dissertation, our group has performed studies applying photochemically crosslinking techniques to enhance the mechanical and degradative properties of hydrogels, while maintaining favorable cell viability and cartilage matrix production.

To advance hydrogel-based biomaterials, a wide variety of stimuli responsive hydrogels have been developed to control their properties with external stimuli (*e.g*. cross-link density, hydrophobicity, swelling rate, permeability, degradability and mechanical strength). A large variety of stimuli-responsive hydrogel systems have been developed that can respond to temperature, pH, light, and other stimuli. Among these stimuli, light is a particularly interesting option as it is a remote stimulus that can be controlled both spatially and temporally with great ease and convenience[234]. Compared to chemical and physical crosslinking, photochemical crosslinking possesses several important advantages. First, the photochemical process has many controllable parameters including laser energy, power density, and photosensitizer concentration. Second, spatial control over the photochemical crosslinking process can be exerted. Selectivity is achieved as photochemical crosslinking only occurs when both light and photosensitizer present and when the target molecules are at the proximity of the photosensitizer and light. Third, temporal control over the process can be exerted as well. Reaction

can be triggered by combining the photosensitizer with the target molecules in darkness and then switching on the light source, or reaction can be easily terminated by turning off the light source at any time. Fourth, photochemical crosslinking is a rapid and efficient process. The duration of the process usually ranges from seconds to minutes. Finally, photochemical crosslinking has lower cell toxicities compared with some other crosslinking methods[55].

In this group of studies, we applied photochemically crosslinking techniques on collagen based hydrogels. Among numerous kinds of hydrogel candidates, collagen has a number of advantages as a scaffold for cell-free and cell-based tissue repair. It is biocompatible, osteocompatible, adhesive, and it has minimal potential for antigenicity after removal of telopeptides *etc*[107]. Gelatin is a water-soluble protein obtained by the denaturation of collagen, and functionalization of gelatin with unsaturated methacrylamide groups results in gelatin-methacrylamide (gelMA)[178]. Based on our findings, photochemical crosslinking of these protein gels achieved increased gel stability, while retaining cell viability and biocompatibility. Such materials could be valuable as a cell carrier scaffolds for cartilage tissue engineering and regeneration.

Specific aim 2: To test nondegradable/degradable hydrogel composites as scaffold materials for cartilage regeneration (Chapter 7-8).

Articular chondrocytes are exposed to high mechanical loads during normal movement. The low friction of cartilage ensures that joint reaction forces act on it more or less perpendicular to its surface. The joint reaction force can greatly exceed body weight, because it arises primarily from muscle tension, which can be considerable during vigorous physical activity. Under extreme circumstances, muscle forces on lower limb joints can probably rise to 10 X body weight[235]. Cell-seeded, degradable scaffolds are being investigated for the purposes of growing articular cartilage *ex vivo* for subsequent implantation[236]. However, even with mechanical and biological stimulation of the constructs, the mechanical properties of the tissues produced thus far are markedly inferior to that of the native tissue[237]. It is questionable whether such materials could withstand physiological joint loads[238].

Less mechanically challenged, but the auricle being one of the most complex three-dimensional structures of the external body[239] poses a very different challenge for which hydrogel composition could be beneficially modified. Unlike articular cartilage repair, the primary goal of auricular reconstruction would be the acquirement of favorable cosmetic appearance instead of functional restoration. During the past two decades, attempts have been reported by various research groups[86, 91, 97-101, 240, 241] that have uncovered difficulties related to engineering three-dimensional human ear-shaped cartilage with its complex architecture and largely unsupported, protruding, three-dimensional structure. The biggest challenge, however, remains to demonstrate specific shape retention of the auricle in longer term *in vivo* studies. Shape changes inevitably occurred upon degradation of the internal supporting polymer scaffold[97, 99], or removal of external stents, which were preserving auricle shape[36]. Numerous biodegradable scaffold materials, including fibrin glue, alginate, porous polymer scaffolds and others, when combined with chondrocytes have been useful in generating new cartilage matrix. However, almost all of these materials do not have suitable mechanical properties to withstand the stress imposed by the surrounding tissues. Also, many of these degradable scaffold materials degrade too fast to allow enough extracellular cell matrix to mature into a suitable, mechanically sound construct to be able to withstand the contraction forces imposed by the skin, especially in large animal models. Cao *et al*[91] reported the first tissue engineered human ear-shaped auricle cartilage using a polyglycolic acid-polylactic acid scaffold in mice for 12 weeks in 1997, which was a milestone in ear regeneration employing tissue engineering techniques. Progress since that report has stalled because scaffold materials are not ideal making it impossible to maintain the ear shape over longer times, even in immunodeficient animal models. Hence, an ideal auricular framework should be durable and retain its shape constantly throughout one's life time.

Nondegradable materials have long been suggested as an alternative to cartilage grafting or engineered tissue since the mechanical properties can be more easily controlled[198]. Nondegradable hydrogel scaffolds (hydrophilic, crosslinked, hydrated, polymeric networks) have demonstrated promising *in vivo* animal model results although an inability to integrate with the surrounding tissue

has been problematic[198]. The major cause would be the acellular-nondegrabale scaffold is only the simple replacement of the tissue, instead of the regeneration of the tissue. Hence, we believed that the rational combination of nondegradable hydrogels, which could be resistant to erosion and maintain three-dimensional architecture, with degradable hydrogels, which could provide a favorable environment for cells to produce ECM, would be leading to a promising strategy for cartilage repair and regeneration.

Poly(vinyl alcohol) (PVA) is a hydrophilic nondegradable hydrogel, the mechanical properties of which can be readily controlled to provide the necessary mechanical support for the use in diarthrodial joints. Several studies have evaluated solid, non-porous PVA as a candidate material for chondral or osteochondral defects in animal models; but integration of the implant with adjacent articular cartilage was absent, threatening the long-term functional ability of the implants[242]. Engineering articular neocartilage using fibrin gel (FG) has been extensively studied in the past by our group[30, 57, 60, 76, 79]. By mixing equal volumes of fibrinogen and thrombin, FG can fill irregularly shaped cartilage defects, and as chondrocytes deposit ECM, neocartilage can form and integrate with native tissues[53]. The results presented in Chapter 7 showed that porous PVA in combination with FG and chondrocytes provides a favorable microenvironment for tissue engineering of articular cartilage. The results from the mechanical testing suggested that this type of biosynthetic construct can mimic the properties of native cartilage and that it can integrate with cartilage. Although this study employed articular chondrocytes for the study, similar results might be obtained for auricular chondrocytes or MSCs in an attempt to generate a stable and durable ear replacement.

Over the past few decades, poly(ethylene glycol) (PEG) hydrogels have been extensively used as matrices for controlling drug delivery, as well as cell delivery vehicles for promoting tissue regeneration. The versatility of the PEG macromer chemistry, together with its excellent biocompatibility, has spurred the development of numerous intelligently-designed hydrogel systems for regenerative medicine applications[243]. The purpose of this study was to evaluate the cartilage formation capacity of a series of polymers made with a combination of degradable and nondegradable PEG macromers. The results showed that articular cartilage regeneration could be achieved using swine articular chondrocytes photoencapsualted into PEGDM copolymer hydrogels, and the neocartilage tissue possessed the ability to integrate with existing adjacent native cartilage. We believe our scientific discoveries based on nondegradable PVA/degradable FG and nondegradable/degradable PEG macropolymers have shown promise for the application of these composited hydrogels for cartilage repair and regeneration.

Concise implications of the "hydrogels" part:

Hydrogels are useful in tissue engineering as they present cells a three-dimensional context for tissue formation and defect repair. These water-swollen networks provide a local microenvironment that can signal to cells through various chemical and mechanical signals and serve as a permeable matrix for the diffusion of soluble factors[244]. However, limited mechanics to withstand physiological loads may be the major drawback to using hydrogels for cartilage repair and regeneration.

These studies demonstrated two efficient strategies, including the application of photochemically crosslinking hydrogels and the combination of nondegradable/degradable hydrogels, to strengthen the hydrogel scaffolds without decreasing the cell viability and support the neocartilage matrix formation. These discoveries might speed the process of applying hydrogel scaffolds combined with appropriate cell sources for repair cartilage in patients. These novel strategies will not only benefit articular cartilage repair in orthopaedics, but also improve auricular reconstruction in plastic and reconstructive surgery. Actually, the preliminary study using nondegradable/degradable hydrogel composite (PVA/alginate) has been already reported by our group[191]. Based on our experience, the first advantage of PVA hydrogel material is its ability to retain shape and contour in the long term. Second, we believe the human ear replacement should be flexible and pliable to provide a naturally feeling ear. Current non-degradable scaffold materials like Medpor® are inflexible and stiff. PVA scaffold can be tailored to be flexible and also can be modified with flexibility and stiffness to simulate the human native external ear. Third and also more importantly according to our published results, porous PVA could provide a favorable environment to permit extracellular matrix formation using a degradable

hydrogel (fibrin glue or alginate) to encapsulate chondrocytes. Hence, a biological hydrogel composite using a non-degradable PVA ear-shape scaffold with a degradable hydrogel to encapsulate cells generating cartilage matrix in the interstices of the scaffold would be ideal for craniofacial reconstruction. This technique for ear regeneration could result in an alloplastic biological hybrid structure with favorable flexibility and pliability that more closely resembles the native auricle.

CONCLUSIONS AND FUTURE PERSPECTIVES

A large body of literature exists on the generation of cartilage through tissue engineering, most of which are *in vitro* studies. The development of cell/scaffold-based therapies continues to be an area of intensive research. This dissertation is a synthesis of much that has been learned, and it focused on generating functional new cartilage using cells, articular, auricular, and mesenchymal stem cells (MSCs), and the manipulation of hydrogels to act as cell carrier devices for cartilage repair and regeneration. The goal was to move beyond *in vitro* assessment and perform these studies *in vivo* in order to move tissue engineering strategies closer to clinical application. The studies presented in this dissertation could provide a sound basis for further translation of cell/scaffold therapies in patients needing cartilage repair.

Based on the clinical results that have been reported thus far, autologous chondrocytes continue to be the favored cell type for cartilage repair and regeneration. In this dissertation, the knowledge gained from cartilage generation using juvenile animal cells was translated into similar results using human cells combined with a hydrogel system. The results have demonstrated that cells from middle-aged, or even older, people have the capacity to generate cartilage ECM that is comparable to that observed by cells from juvenile animals—which has been the focus of most reported experimental cartilage engineering studies. Evaluating these parameters will help in developing strategies to apply tissue engineering clinically.

Due to the limitations caused by donor site morbidity and loss of phenotype during *in vitro* expansion when using chondrocytes, mesenchymal stem cells are a promising alternative cell source that needs to be explored. In this dissertation, experiments have shown that chondrocyte-conditioned medium (CCM) was effective in inducing chondrogenic differentiation of BMSCs resulting in new cartilage ECM. Specifically, CCM had a stronger influence on chondrogenesis than supplementation of the standard culture medium with TGF-β3 without inducing calcification. The further identification of factors in CCM that help improve chondrogenesis of MSCs and prevent ossification remains to be evaluated. Undoubtedly, the elucidation of these molecules and their mechanism of action will provide an appropriate strategy for improving chondrogenic cell therapies from MSCs.

Among the numerous scaffolds, hydrogels could be ideal cell carriers for cartilage tissue engineering. In this dissertation, results have shown that the photochemically crosslinked hydrogels and the combination of nondegradable/degradable hydrogel composites have great potential to exceed the traditional natural hydrogels to be better scaffolds for cell-based cartilage repair and regeneration. Since all of the cells used for this work were harvested from swine, human cells including chondrocytes and mesenchymal stem cells grown in conditioned medium will be combined with these advanced hydrogels to achieve the translation for the next steps.

The interpretation of these studies provides strong evidence for performing large animal experiments and clinical trials using tissue engineering techniques. Cell/hydrogel implantation applying the strategies demonstrated in this dissertation into immunocompetent animal models and long-term outcomes will be tested in the near future. Ultimately, clinical translation and feasibility needs to be considered with all of these approaches if a successful tissue engineered cartilage product is to make it through the regulatory process and into patients.

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DUTCH SUMMARY / NEDERLANDSE SAMENVATTING

Het eerste doel van dit proefschrift is de evaluatie van verschillende celtypes voor kraakbeenregeneratie. De geteste celtypen omvatten chondrocyten en mesenchymale stamcellen (MSCs) gecombineerd met biomimetische scaffold materialen.

Doelstelling 1: Het onderzoeken van kraakbeenregeneratie met volwassen humane chondrocyten gezaaid in een biologische hydrogel (hoofdstuk 2).

De resultaten van deze studie toonden dat chondrogenese door chondrocyten uit gewrichten van volwassen mensen op een voorspelbare en betrouwbare wijze kan worden gerealiseerd. Hoewel de uitkomsten niet identiek zijn aan het kraakbeen gemaakt door juveniele varkenschondrocyten, waren er veel overeenkomsten in histologie, druksterkte, en de productie van collageen. Omdat de meeste patiënten met kraakbeendefecten volwassen zijn, is de vergelijking van juveniele varkenschondrocyten met volwassen humane chondrocyten belangrijk voor de translatie naar de kliniek. Hoewel de onderzoeksopzet tevens groepen met juveniele humane cellen of oudere dierlijke cellen had kunnen includeren, was dit logistiek niet mogelijk wegens de sterke beperking in beschikbaarheid van deze cellen. Verder is het doel van deze studie om te bepalen of cellen van volwassenen mensen het vermogen hebben om kraakbeenmatrix te genereren die vergelijkbaar is met die geproduceerd door cellen van jonge dieren. De laatste groep wordt namelijk veel gebruikt in studies naar de regeneratie van kraakbeen. De evaluatie van deze parameters zal bijdragen in het ontwikkelen van strategieën om tissue engineering benaderingen toe te passen in de kliniek.

Doelstelling 2: Het onderzoeken van kraakbeenregeneratie met dierlijke chondrocyten en mesenchymale stamcellen (BMSCs) gekweekt in geconditioneerd medium, dat verzameld is van gekweekte chondrocyten, en gecombineerd met biologische dragerstructuren (hoofdstuk 3-4).

Het eerste doel van deze experimenten was om het elastische kraakbeenweefsel van het oor te maken met dierlijke chondrocyten gezaaid op een biologische dragerstructuur van collageen. Waar acute defecten in het oppervlak van een gewricht opgevuld kunnen worden met cellen of dragerstructuren, omvat de reconstructie van het oor in het algemeen de creatie van de gehele externe oorschelp. De uitdaging hierin is om voldoende hoeveelheden kraakbeen te regenereren dat een complex gevormde driedimensionale vorm kan behouden in een onderhuids wondbed. Verscheidene eerdere studies hebben hydrogelen gecombineerd met dierlijke chondrocyten om oorkraakbeen te genereren; echter zijn hydrogelen mechanisch zwak en vereisen zij externe ondersteuning om de driedimensionale architectuur van het oor te behouden. Om deze reden werd in deze studie gekozen voor een fibreuze dragerstructuur van collageen type I/III, welke zo gemanipuleerd en versterkt kan worden zodat het construct de krachten van de overliggende huid kan weerstaan. De resultaten van deze studie tonen aan dat nieuw oorkraakbeen *in vivo* gegenereerd kan worden met deze fibreuze dragerstructuur gezaaid met dierlijke kraakbeencellen uit het oor.

Het tweede doel van deze experimenten was om middelen te onderzoeken om de gezaaide dragerstructuren voor te kweken om het kraakbeenvormend vermogen na *in vivo* implantatie te verbeteren. De vorming van kraakbeenmatrix door de cellen op de drager wordt vergeleken onder twee condities, namelijk statisch en dynamisch gekweekt voorafgaande aan implantatie in proefdieren. Het voorkweken van cellen op de dragers onder dynamische condities toonde een licht verbeterde kraakbeenformatie in vergelijking met de resultaten van een statische kweek, wat het gevolg kan zijn van de stimulatie van kraakbeenvorming voorafgaand aan implantatie.

Concluderend zorgen bovenstaande condities voor een uniforme kraakbeenvorming. Echter kan de beperkte beschikbaarheid van autologe kraakbeencellen uit het oor deze strategie limiteren. Daarom zijn betrouwbaardere en praktischere celtypen en strategieën ontwikkeld worden voor kraakbeenherstel en regeneratie.

Mesenchymale stamcellen zouden een uitstekende bron kunnen zijn als zij in voldoende aantallen geoogst kunnen worden en kunnen worden gestuurd richting chondrogene differentiatie. Een van de grootste uitdagingen in het gebruik van mesenchymale stamcellen uit het beenmerg (BMSCs) voor de

generatie van kraakbeen is het induceren van chondrogenese. Transforming growth factor-beta 3 (TGF- β3) heeft laten zien een van de meest efficiente groeifactoren te zijn voor dit doel. Echter kunnen mesenchymale stamcellen een hypertrofisch fenotype krijgen bij chondrogene inductie, wat resulteert in de vorming van calcificaties na implantatie in het lichaam. Daarnaast is de klinische toepassing van exogene recombinante groeifactoren nog niet bewerkstelligd. Co-kweek strategieën zouden een oplossing kunnen bieden aan bovenstaande uitdagingen doordat hierin de cellen worden blootgesteld aan meerdere natuurlijke stimuli. Het belangrijkste doel van dit deel van de studie was om te bepalen of geconditioneerd medium, verzameld van gekweekte chondrocyten uit het oor, de chondrogene differentiatie van mesenchymale stamcellen en dus kraakbeenvorming kunnen bevorderen. Het stimulerend potentieel van geconditioneerd medium werd vergeleken met het standaard protocol voor de chondrogene differentiatie van mesenchymale stamcellen: de toevoeging van exogeen TGF-β3. Hoofdstuk 4 laat zien dat geconditioneerd medium van chondrocyten effectief is in het induceren van chondrogenese van mesenchymale stamcellen uit het beenmerg (BMSCs), waarbij hogere productie van collageen en uitgebreide kraakbeenformatie werd gezien in vergelijking met standaardkweken. Daarnaast werd alleen botvorming gezien in de groepen met standaardkweek en toegevoegd TGF-β3. Uit deze experimenten concludeerden wij dat geconditioneerd medium een sterkere invloed heeft op de chondrogenese dan de toevoeging van TGF-β3 aan standaard medium, zonder tekenen van endochondrale botvorming. Efficiënte chondrogene differentiatie van BMSCs kan dus een veelbelovende alternatieve celpopulatie zijn voor de regeneratie van kraakbeen. Deze studie is voor zover bekend een van de weinige studies die MSCs in chondrocyte-geconditioneerd medium gebruiken voor de succesvolle regeneratie van oorkraakbeen, welke bovenal werd aangetoond *in vivo*.

Het tweede doel van dit proefschrift is de evaluatie van verschillende hydrogelen als "celdrager" voor kraakbeenregeneratie.

Doelstelling 1: Het onderzoeken van kraakbeenregeneratie middels photochemisch gecrosslinkte hydrogelen (hoofdstuk 5-6).

Er is een grote verscheidenheid aan hydrogelen ontwikkeld die reageren op temperatuur, pH, licht, en andere stimuli. Licht is een bijzonder interessante toepassing omdat een externe stimulus is die zowel ruimtelijk als in de tijd kan worden gestuurd met groot gemak en comfort.

Het eerste doel van deze studie is om kraakbeen te regenereren middels dierlijke chondrocyten uit het gewrichtsoppervlak, welke vervolgens werden ingekapseld in photochemisch gecrosslinkte hydrogelen. Hoofdstuk 5 onderzocht het 'duaal' crosslinken van een collageen hydrogel door (a) crosslinking met Rose Bengal (RB) en groen licht gevolgd door (b) chemische crosslinking met 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) en N-hydroxysuccinimide (NHS), met als doel de stijfheid van de collageen gel te verbeteren. Hoofdstuk 6 heeft aangetoond dat een gelatine-methacrylamide (gelMA) hydrogel een gunstige drager is voor zowel articulaire als auriculaire chondrocyten, gezien de productie van extracellulaire matrix. Op basis van deze data blijkt dat photochemische crosslinking van zulke hydrogels leidt tot verhoogde stabiliteit van de hydrogel, terwijl ook de biocompatibiliteit van de gel en de levensvatbaarheid van de cellen behouden blijft. Zulke materialen kunnen waardevol zijn als celdragers voor de generatie van kraakbeenweefsel.

Doelstelling 2: Het testen van verschillende "biomimetische" hydrogel composieten als celdragers voor kraakbeenregeneratie (hoofdstuk 7-8).

De resultaten uit hoofdstuk 7 en 8 laten zien dat hydrogelen samengesteld uit niet-afbreekbare (poly(vinylalcohol) (PVA)) en een afbreekbare fibrine hydrogel een gunstige omgeving is voor kraakbeenregeneratie. Mechanische tests suggereren dat zulke biosynthetische constructen de eigenschappen van het natuurlijke weefsel kunnen nabootsen en dat het kan integreren met kraakbeen. Hoewel deze studie specifiek chondrocyten uit het gewricht heeft gebruikt, zouden soortgelijke

resultaten behaald kunnen worden voor chondrocyten uit het oor of mesenchymale stamcellen om een stabiel en duurzaam oorimplantaat te maken.

Het doel van hoofdstuk 8 was om de kraakbeenvormende capaciteit van verschillende copolymeren, bestaande uit afbreekbare en niet-afbreekbare PEG macromeren, te evalueren. Onze resultaten laten zien dat vorming van gewrichtskraakbeen behaald kon worden middels het gebruik van articulaire chondrocyten in een hydrogel van PEG copolymeren. Daarbij bezat het nieuwe kraakbeenweefsel de capaciteit om te integreren met het bestaande aangrenzende kraakbeen.

Op basis van deze ontdekkingen kunnen we aannemen dat een rationele combinatie van niet-afbreekbare hydrogelen – die bestand zijn tegen erosie en de driedimensionale architectuur van het construct kunnen behouden, en afbreekbare hydrogelen – die een gunstige omgeving voor cellen verschaffen voor de productie van extracellulaire matrix, kan leiden tot een veelbelovende strategie voor kraakbeenherstel en regeneratie.

Conclusies en toekomstperspectieven

Er bestaat een grote hoeveelheid literatuur over de generatie van kraakbeen middels tissue engineering, waarvan de meeste *in vitro* studies zijn. De ontwikkeling van celdrager-gebaseerde therapieën blijft een gebied van intensief onderzoek. Dit proefschrift is een samenstelling van veel dat is geleerd, en richt zich op het genereren van functioneel nieuw kraakbeen middels het gebruik van articulaire chondrocyten, auriculaire chondrocyten, en mesenchymale stamcellen, en de manipulatie van hydrogelen die functioneren als dragers voor kraakbeenherstel en regeneratie. Het doel was om verder te gaan dan *in vitro* evaluaties en de experimenten *in vivo* uit te voeren om zo tissue engineering strategieën dichterbij klinische toepassing te brengen. De studies in dit proefschrift zouden een goede basis kunnen zijn voor verdere vertaling van celdragende therapieën om kraakbeenherstel aan te bieden aan patiënten.

Gebaseerd op de klinische resultaten die tot nu toe zijn gerapporteerd, hebben autologe chondrocyten als celbron nog steeds de voorkeur voor kraakbeenherstel en regeneratie. In dit proefschrift wordt de opgedane kennis van kraakbeenregeneratie met juveniele dierlijke cellen vertaald naar vergelijkbare resultaten met menselijke cellen in combinatie met een hydrogel systeem. De resultaten hebben aangetoond dat cellen afkomstig van mensen van middelbare leeftijd, of zelfs ouder, het vermogen hebben om nieuw kraakbeen te vormen dat vergelijkbaar is met de resultaten uit studies die juveniele dierlijke cellen gebruiken. De evaluatie van deze parameters zal helpen bij het ontwikkelen van strategieën om tissue engineering klinisch toepasbaar te maken.

Vanwege de beperkingen veroorzaakt door donorplaats morbiditeit en verlies van fenotype tijdens *in vitro* expansie bij het gebruik van autologe chondrocyten, zijn mesenchymale stamcellen een veelbelovend alternatief. In dit proefschrift hebben experimenten aangetoond dat chondrocyt-geconditioneerd medium effectief is in het induceren van chondrogene differentiatie van mesenchymale stamcellen uit beenmerg, resulterend in nieuw kraakbeen. Geconditioneerd medium had een sterkere invloed op de chondrogenese dan suppletie van standaard medium met TGF-β3, zonder verkalking te induceren. De verdere identificatie van factoren in het geconditioneerde medium die bijdragen aan de chondrogenese moet nog worden geëvalueerd. Ongetwijfeld zal de opheldering van deze moleculen en hun werkingsmechanisme een geschikte strategie ter verbetering van chondrogene celtherapie middels mesenchymale stamcellen verschaffen.

Onder de talrijke celdragers, zijn hydrogelen ideale mobiele dragers voor kraakbeenregeneratie. In dit proefschrift hebben de resultaten laten zien dat photochemisch gecrosslinkte hydrogelen alswel de combinatie van niet-afbreekbare met afbreekbare hydrogel composieten grote potentie tonen om de traditionele natuurlijke hydrogels te passeren als drager in cel-gebaseerd kraakbeenherstel en regeneratie. Aangezien alle cellen in deze experimenten van dierlijke oorsprong waren, zullen humane cellen – waaronder chondrocyten en mesenchymale cellen gekweekt in geconditioneerd medium – combineerd worden met deze geavanceerde hydrogelen om zo de vertaling van de volgende stappen te realiseren.

De interpretatie van deze studies levert sterk bewijs voor het uitvoeren van grote dierproeven en klinische trials met tissue engineering technieken. Het toepassen van de implantatie van cel/hydrogelconstructen zoals beschreven in dit proefschrift zullen in immunocompetente diermodellen en met langetermijn uitkomsten zal in de nabije toekomst uitgevoerd worden. Uiteindelijk zal de klinische vertaling en haalbaarheid moeten worden beschouwd met al deze benaderingen als een succesvol tissue-engineered kraakbeen product goedkeuring krijgt om in patiënten toepassing te vinden.

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CURRICULUM VITAE

The author of this dissertation was born on June 14th 1981, in Shijiazhuang and Hebei Province, P. R. China. In 1999 he graduated from Shijiazhuang No. 2 middle school, one of the key high schools in China, and started receiving his medical education in Xiangya School of Medicine, Central South University, China, which was cofounded by Yale University one century ago. During this period, he won "Excellent Student Leader Award" every year.

After Dr. Zhao graduated summa cum laude in 2004 from medical school, he was chosen for the only residency position among the numerous applicants throughout the country by Plastic and Reconstructive Surgery at the Zhongshan Hospital and Fudan University, Shanghai, one of the most prestigious hospitals in Asia.

After 3 years' surgical training, Dr. Zhao was offered a precious position as postdoctoral research fellow working for both Plastic Surgery Research Laboratory and Laboratory for Musculoskeletal Tissue Engineering, Orthopaedic Surgery at the Massachusetts General Hospital and Harvard Medical School, Boston, U.S. in August, 2007. Over the past more than 7 years, he has shown to be an extraordinary investigator responsible for significant advances in the field of tissue engineering and regenerative medicine, especially on cartilage repair and regeneration. Dr. Zhao has been a leading researcher working on several vital projects funded by the U.S Armed Forces Institute of Regenerative Medicine (AFIRM) and Department of Defense. As one of the most successful research fellows in the past decade's history in his lab, Dr. Zhao was promoted to be a junior faculty member and appointed as Instructor of Orthopaedic Surgery by the Harvard Medical School in March, 2011.

In the summer of 2013, Dr. Zhao started pursuing his Ph.D. degree in Department of Orthopaedics, University Medical Center Utrecht following Prof. dr. D.B.F. Saris, Prof. dr. W.J.A. Dhert, and Dr. ir. J. Malda. His project has been focused on the application of different cell sources and multiple biomimetic hydrogel scaffolds for cartilage regeneration. So far, Dr. Zhao has authored or coauthored 13 papers and 2 book chapters, and several more will be released in the coming months. He has been invited to give oral presentations at various international and domestic conferences more than 40 times. In 2010, he won First Place in the U.S. Plastic Surgery Foundation Scientific Essay Contest for his ear reconstruction paper (first author) and American Society of Maxillofacial Surgeons/Maxillofacial Surgeons Foundation (ASMS/MSF) Research Grant Award (co-author). As a young investigator, Dr. Zhao has been invited as an active reviewer for up to 14 prestigious journals such as Plastic and Reconstructive Surgery (PRS), American Journal of Sports Medicine, Tissue Engineering *etc* in the past several years. Since January, 2013, he has been working as an Associate Editor of BMC Muscoloskeletal Disorders (BioMed Central). After achieving his Ph.D. degree, Dr. Zhao will be pursuing his residency training in orthopaedic surgery in the United States.

Dr. Zhao lives together with his wife Dr. Ying Zhou. They have one lovely daughter, Lexie.