

Proliferative, Contractile, and Biosynthetic Activity of Adult Canine Articular Chondrocytes in Type I and II Collagen-Glycosaminoglycan Matrices *In Vitro*

by

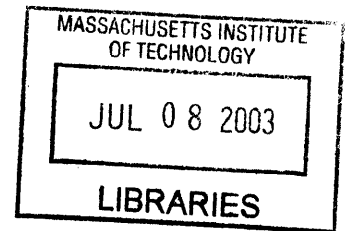
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B.S. Mechanical Engineering
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Submitted to the Department of Mechanical Engineering
in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Mechanical Engineering
at the
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BARKER

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ABSTRACT

One tissue engineering approach being investigated for the treatment of defects in articular cartilage involves the implantation of autologous chondrocyte-seeded absorbable scaffolds. The purpose of this thesis was to establish a foundation for future studies by investigating the behavior of freshly isolated and passaged chondrocytes in scaffolds of different composition.

This study evaluated the effects of passage number (freshly isolated and passages 1 and 2) and collagen type on the proliferation, contraction, and biosynthetic activity of adult canine articular chondrocytes grown in type I and II collagen-glycosaminoglycan matrices. Adult articular chondrocytes were acquired from 8 canines, and either freshly isolated cells or cells cultured in monolayer up to two passages were seeded into porous type I and type II collagen-glycosaminoglycan matrices (CG). These porous scaffolds were manufactured by freeze-drying and were subsequently cross-linked by dehydrothermal (DHT) and carbodiimide (EDAC) treatments. The matrices were cut to disks 4mm in diameter by 3mm thick and were dynamically seeded at a cell density of 10,000 chondrocytes/mm³. The type II CG matrices were terminated at 1-day and 1, 2, 4, 8, and 12 weeks in culture. Culture of the type I matrices was terminated after 1-day and 1 and 4 weeks.

EDAC cross-linking imparted a degree of mechanical stiffness to the matrices such that they adequately resisted the smooth muscle actin-enabled contraction. P0, P1, and P2 cells seeded in the type II matrices continued to proliferate over a 4-week period, but thereafter the P0 and P1 cells continued to increase in number and the P2 cells decreased. It was demonstrated that collagen type (type I or type II) did not have a significant effect on DNA content, GAG content, or biosynthesis for up to 4 weeks when all data were included. Due to the variance in data, statistical outliers were excluded and statistical analyses revealed a significant effect of collagen type on protein and GAG synthesis with higher levels in the type II scaffold. Western blot analysis demonstrated that P1 and P2 cells seeded in the type II matrices were capable of synthesizing type II collagen thus demonstrating that they retained the articular chondrocyte phenotype. These findings support the continued investigation of type I or II CG matrices seeded with second passage chondrocytes and culture for 4 weeks as implants for articular cartilage tissue engineering. The advantage of being able to culture chondrocytes for 2 passages is that greater numbers of cells can be made available for seeding purposes.

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CHAPTER 1: INTRODUCTION

1.1 Statement of the Problem

The loss or degeneration of articular cartilage is a serious problem that plagues millions. Focal lesions in articular cartilage, that arise from either trauma, disease, or continual mechanical loading, if left untreated can lead to great pain and instability for an individual with the potential of becoming debilitating. Isolated chondral and osteochondral defects' histories are not well defined [3-5], clinical experience has shown that when left untreated, these lesions fail to heal and that defects that involve a major portion of the articular surface may progress to symptomatic degeneration of the joint. A solution being explored for this problem is an implant to assist in the regeneration of articular cartilage.

The implant being explored in this thesis, is an autologous chondrocyte-seeded collagen-glycosaminoglycan (CG) matrix synthesized from type I or II collagen. These matrices have been cultured under specific conditions *in vitro* in an effort to determine the effects of passage number, collagen type, and time in culture on the proliferative and biosynthetic activity of the seeded articular chondrocytes. For this experiment, passage number and time were explored in attaining these objectives.

1.2 Articular Cartilage

Articular cartilage is the connective tissue covering the ends of bones at the articulating surface of synovial joints with the ability to bear loads. The surface of articular cartilage is nearly frictionless allowing the ability to move around smoothly and in the absence of pain. Figure 1.1 displays the anatomy of a human knee and location of articular cartilage.

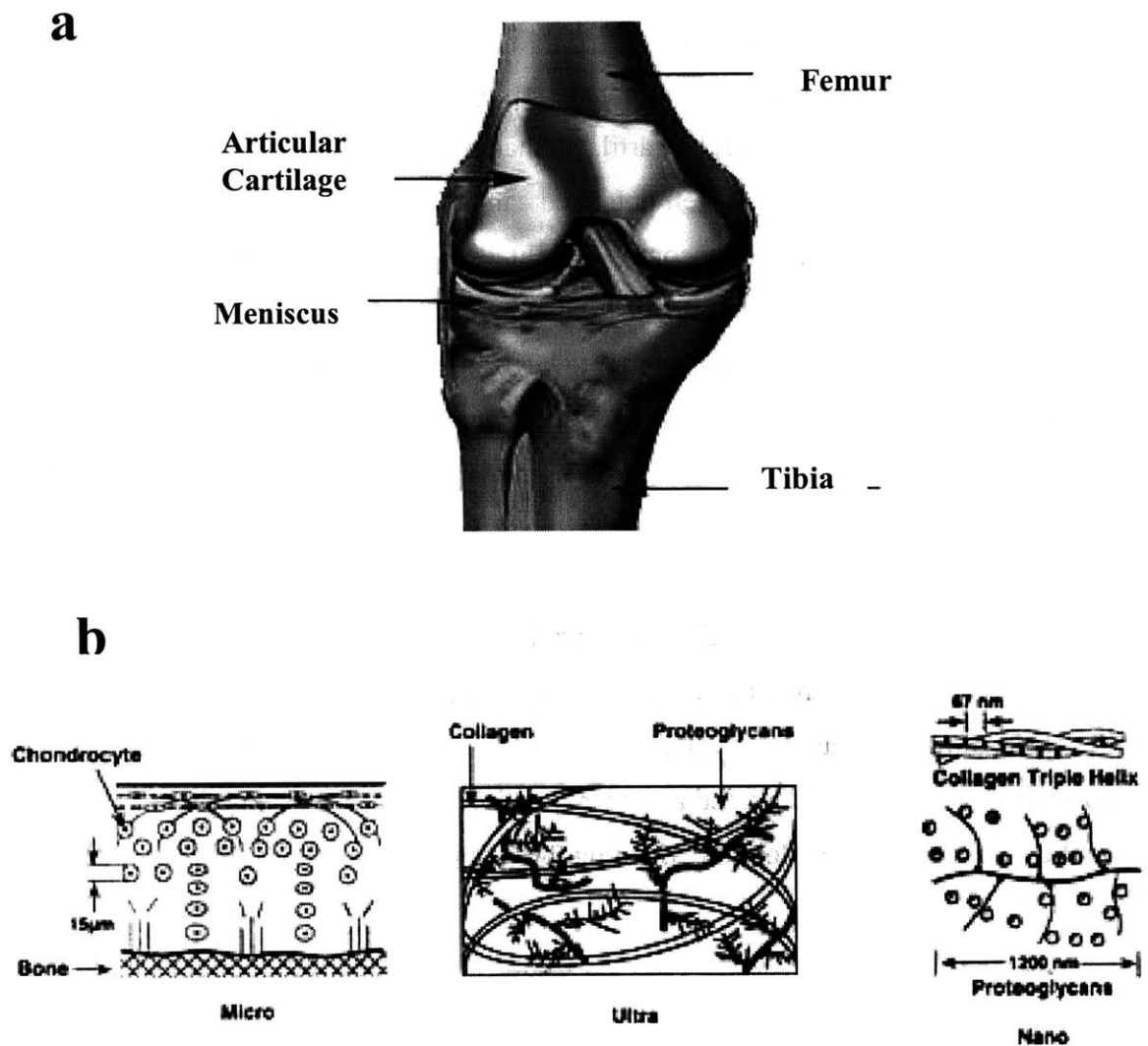


Figure 1.1. Anatomy of articular cartilage. (a) Anatomy of the human knee displaying location of articular cartilage. (b) Composition of articular cartilage at varying magnifications, microstructural level $0.1\mu\text{m} - 100\mu\text{m}$, ultrastructural level $0.01\mu\text{m} - 1\mu\text{m}$, and nanostructural level $0.1\text{nm} - 1\text{nm}$. Images from Basic Orthopaedic Biomechanics [2].

1.2.1 Composition and Function

The chondrocyte is the only type of cell that makes up normal articular cartilage, but contributes little to the volume of the tissue accounting for only 1% in adult human articular cartilage [6]. A spherical shape, synthesis of type II collagen, large aggregating proteoglycans, and specific non-collagenous proteins, and formation of these molecules

into cartilaginous matrix are the characteristics that distinguish the chondrocyte from other cells. Individual chondrocytes are active metabolically, but the total metabolic activity of the tissue is low because of the low cell density. Chondrocytes derive their nutrition from the nutrients in the synovial fluid, which to reach the cell, must pass through a double diffusion barrier: first the synovial tissue and synovial fluid, and then the cartilage matrix.

Chondrocytes surround themselves with their extracellular matrix (ECM) and do not form cell-to-cell contacts. The tissue itself is recognizable as cartilage from an accumulation of matrix that separates the cells, and they assume a spherical shape. Water contributes to approximately 80% of the wet weight of articular cartilage with the dry weight being accounted for by 60% collagen, 25-35% proteoglycans, and 15-20% non-collagenous proteins and glycoproteins. Chondrocytes form the macromolecular framework of the tissue matrix from three classes of molecules: collagens, proteoglycans, and non-collagenous proteins.

Articular cartilage contains multiple collagen types, specifically types II, VI, IX, X and XI [7]. Type II collagen accounts for 90-95% of the collagen in articular cartilage and forms the primary component of the cross-banded fibrils. Type X collagen is located only near the cells of the calcified cartilage zone of the articular cartilage and the hypertrophic zone of the growth plate, suggesting it has a role in the mineralization of the cartilage.

Two major classes of proteoglycans make up articular cartilage: large aggregating proteoglycan monomers or aggrecans, and small proteoglycans including decorin, biglycan, and fibromodulin [8-10]. Aggrecans have a large number of chondroitin sulfate and keratan sulfate chains attached to a protein core filament. Aggrecan molecules fill most of the interfibrillar space of the cartilage matrix, 90% of the total cartilage matrix proteoglycan mass. Glycosaminoglycans found in cartilage include hyaluronic acid, chondroitin sulfate, keratan sulfate, and dermatan sulfate. Large aggregating proteoglycans (aggrecans) give the tissue its stiffness to compression and its resilience and contribute to its durability.

Because of the importance of type II collagen in articular cartilage one aim of this thesis is to evaluate its synthesis by chondrocytes seeded in the collagen matrices.

Moreover, that type II collagen is the principal structural protein in articular cartilage serves as rationale for investigating a type II collagen-GAG scaffold as an analog of extracellular matrix.

1.2.2 Causes of injury, disease, and degeneration

The loss of articular cartilage leads to the symptoms of osteoarthritis (pain and loss of joint function). Osteoarthritis (also referred as degenerative joint disease, degenerative osteoarthritis, osteoarthritis, and hypertrophic osteoarthritis) consists of generally progressive loss of articular cartilage accompanied by attempted repair of the cartilage, remodeling and sclerosis of subchondral bone, and the formation of subchondral bone cysts and marginal osteophytes [11-14]. Changes in the synovial joint, a diagnosis of osteoarthritis requires the presence of symptoms and signs that may include joint pain, restriction of motion, joint effusions, and deformity. Osteoarthritis develops most commonly in the absence of a known cause, less frequently, it develops as a result of joint injury, infection or one of a variety of hereditary, developmental, metabolic, and neurological disorders. Although the symptoms of osteoarthritis, primarily pain and stiffness of the joint, result from degeneration of the joint, the severity of the loss of articular cartilage is not necessarily closely related to the severity of the symptoms.

Most instances, degeneration of articular cartilage and remodeling of subchondral bone are both present when symptoms develop, and it's the loss of articular that leads directly to the loss of joint function. Loss of the larger aggregates appears to be one of the earliest changes associated with osteoarthritis and immobilization of the joint. This loss occurs more frequently with increasing age, possibly because age-related changes in the cartilage matrix and a decrease in the chondrocytic anabolic response compromise the ability of the tissue to maintain and restore itself [15, 16]. The long-term goal of our work is to develop an implant to treat lesions in articular cartilage soon after injury in order to prevent their extension and contribution to more extensive joint degeneration. Such lesions can now be identified using magnetic resonance imaging.

1.2.3 Healing of Articular Cartilage

Repair refers to the restoration of a damaged articular surface with new tissue that resembles but does not duplicate the structure, composition, and function of articular

cartilage; regeneration refers to the formation of new tissue indistinguishable from normal articular cartilage [17]. Studies have shown that loading and motion influences the healing of articular cartilage and joints [18] and that mechanical loading influences the repair process in all tissues that form parts of synovial joints [19]. A variety of methods have the potential to stimulate the formation of new articular surface, but the available evidence indicates that the results vary considerably among individuals and that the tissue that forms after treatments doesn't duplicate the composition, structure or mechanical properties of normal articular cartilage. It is also noted that isolated defects of articular cartilage and osteochondral defects appear to result from trauma that often leaves most of the articular surface intact [20]. This thesis is to serve as the foundation for work to develop an implant that well facilitates the formation of a functional reparative tissue.

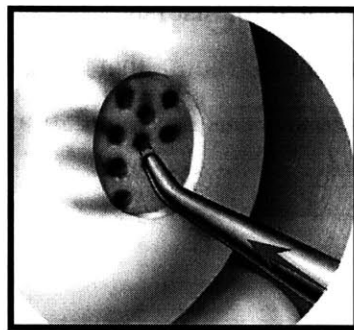
1.3 Clinical background

In the last three decades clinical and basic scientific investigations have shown that implantation of artificial matrices, growth factors, perichondrium, periosteum, and transplanted chondrocytes and mesenchymal stem cells can stimulate the formation of cartilaginous tissue in osteochondral and chondral defects in synovial joints [4, 17, 21]. Limited ability of host cells to restore articular surfaces has led investigators to seek methods of transplanting cells that can form cartilage into chondral and osteochondral defects.

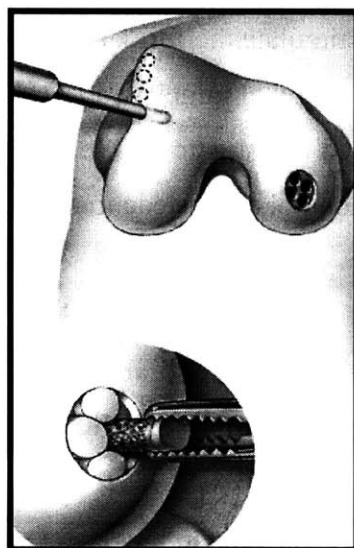
Some current methods for treating focal defects in articular cartilage are microfracture, autologous chondrocyte implantation (ACI), and osteochondral autografting (OCA). When a cartilage lesion penetrates the subchondral bone, blood and stem cells are released and promote filling of the wound with repair tissue. The technique of microfracture (Figure 1.2a) draws the blood and stem cells to the defect site by puncturing the underlying subchondral bone and stimulating the formation of repair tissue in the lesion. Although, the increased foundation of repair tissue may provide pain relief, the results are temporary due to the inferior mechanical properties of repair tissue.

OCA and ACI are transplantation procedures that intend to fill the defect with cartilage synthesized *in situ* by mature cartilage from "non-weight bearing" locations (OCA) or transplanted chondrocytes (ACI). For osteochondral autografting (Figure

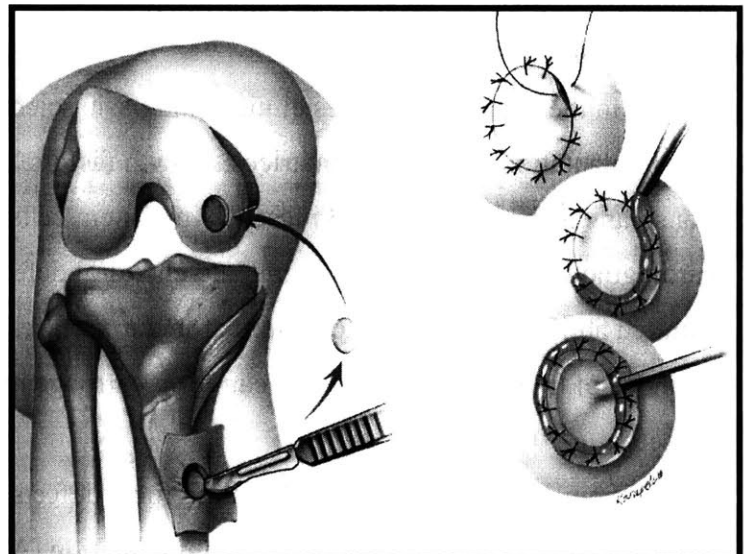
1.2b), autologous grafts can be obtained from the patella, the femoral condyle, and the proximal part of the fibula, but because of the limited possible donor sites, only localized regions of damage articular cartilage can be considered. Autografting results have shown that the surface of articular cartilage can be repaired by this method. Outerbridge, *et al.*, conducted a study treating ten patients with osteochondral defects of the femoral condyle with patellar osteochondral autografts, and found that knee function and symptoms were improved in all patients for an average of 6.5 years after transplantation [22].



a



b



c

Figure 1.2. Clinical procedures for the treatment of articular cartilage defects. (a) Microfracture (b) Osteochondral autografting (c) Autologous chondrocyte implantation. Images from Operative Techniques in Orthopaedics [1].

For the procedure of autologous chondrocyte implantation (Figure 1.2c), chondrocytes are harvested from a "non-weight bearing" location, cultured and expanded in monolayer, and inserted into the focal lesion under a periosteal flap secured by suturing and fibrin glue. Brittberg, *et al.*, has shown that this procedure can alleviate pain for the patient [23]. Although ACI has had encouraging results, and short-term studies (3-months) with rabbits demonstrate similar results to that found in humans, long-term studies in canines have not been as encouraging. The initial procedure incited cartilage repair for canine knees, but 12-18 months after surgery, the quality and robustness of repair tissue began to degrade [24]. Also, in another study [25] for the same time period, no difference was recorded between the healing of the control defect, the defect with a periosteal flap alone, or a defect with autologous chondrocytes under a periosteal flap. Since, it has been established that the repair of canine cartilage is similar to that of humans [26], the long term effectiveness of ACI is questionable.

Even though these clinical procedures may alleviate joint pain and improve joint function [27], no surgical solution has been confirmed as a long term solution as well as having the ability to fully regenerate articular cartilage rather than the formation of repair tissue. For cases in which the wound lesion has progressed to severe degeneration, the current treatment is to replace the entire joint with a prosthesis (Figure 1.3). For this reason, early treatment of cartilage defects, like the methods described above and that serve as a focus of this thesis, may prevent future degradation and future total joint replacement.

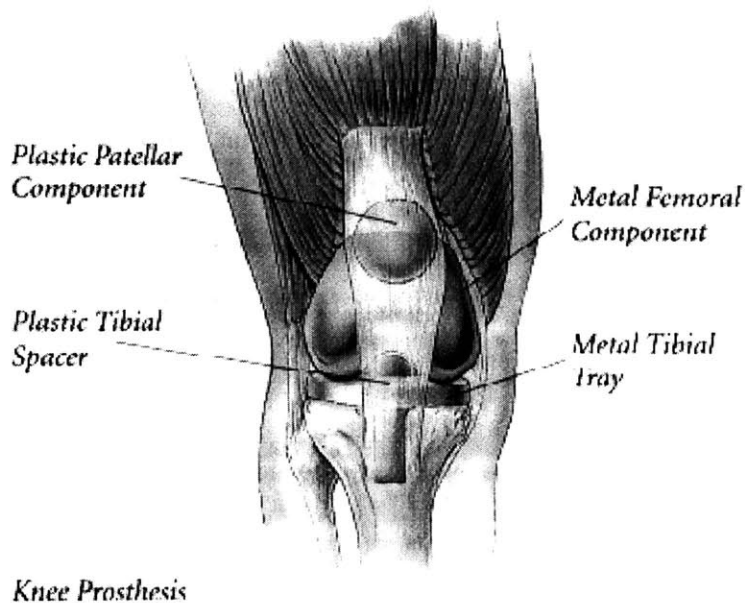


Figure 1.3. Example of a metal/plastic total knee replacement prosthesis.
 Image downloaded from www.ozarkortho.com.

1.4 Tissue Engineering of Articular Cartilage

Better understanding of the lesions and degeneration and recognition of the limitations of current treatments have contributed to the recent interest in the repair and regeneration of cartilage. A lack of data makes it difficult to compare the relative merits of different types of artificial matrices and to evaluate the possibility that some implanted materials may cause synovitis [28], however what is available indicates that at least some types of artificial matrices can contribute to the restoration of an articular surface. Investigators have found that implants formed from a variety of biological and non-biological materials, including treated cartilage and bone matrices, collagens, collagen and hyaluronan, fibrin, carbon, fiber, hydroxyapatite, porous polylactic acid, polytetrafluoroethylene, polyester, and other synthetic polymers, facilitate the restoration of an articular surface [21].

Since the scaffold serves as an analog of the extracellular matrix, it is important that the scaffold is biocompatible and promotes cell proliferation and matrix synthesis. The composition and porosity of the scaffold as been shown to affect the activity of the cell including maintenance of its phenotype, viability, and biosynthetic activity [29-31].

Numerous synthetic [32-35] and natural materials [36-39] have been employed for the fabrication of the porous absorbable matrix for cartilage tissue engineering. For tissue engineering of cartilage, some of the most commonly used materials are collagen hydrogels [40, 41], agarose [42], sponge-like scaffolds composed of collagen [29-31, 42-44], and polyglycolic acid [30, 45].

A general approach to tissue engineering involves three main areas: 1) the scaffold to serve as an analog of the extracellular matrix, 2) the cells, and 3) regulators and or growth factors [46]. The approach being taken in this thesis entails the use of porous type I and type II collagen-glycosaminoglycan matrices as extracellular matrix analogs (Figure 1.4) that are seeded with adult canine articular chondrocytes. Exploring the effects of chondrocyte behavior in the sited scaffold *in vitro* is an important step towards using the cell-seeded scaffold for implantation.

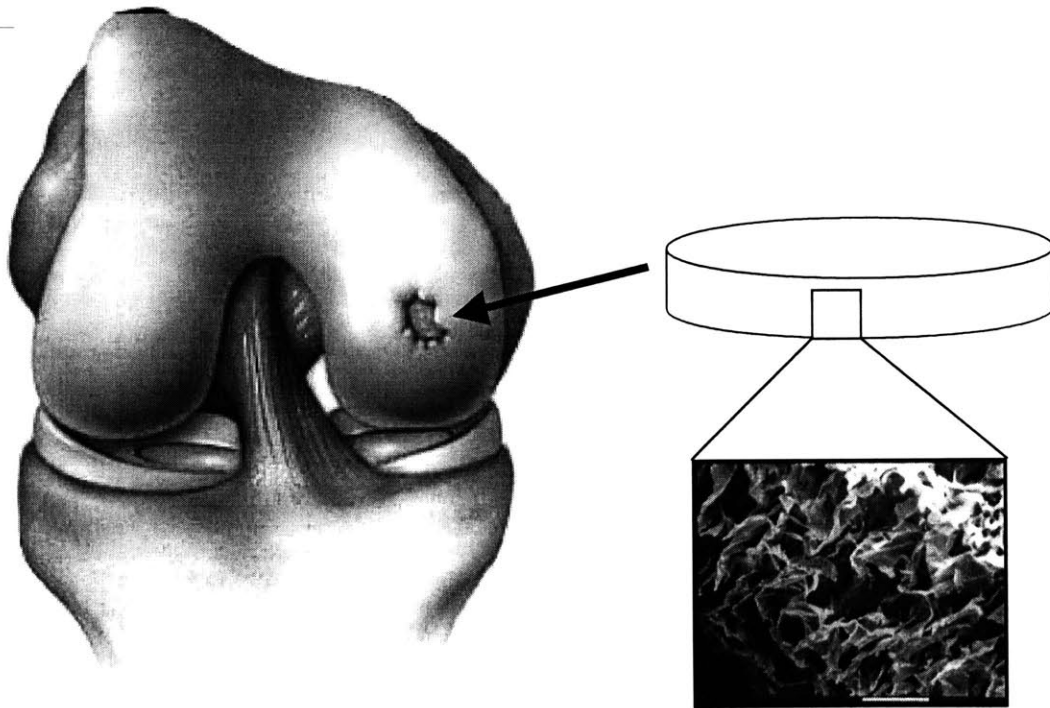


Figure 1.4. Tissue engineering system. Focal lesion in articular cartilage of the knee to be filled with a seeded matrix (disc). Zoomed in view of matrix is a scanning electron microscopic image of a collagen-GAG matrix. Image from Operative Techniques in Orthopaedics [1].

1.5 Specific Aims, Hypotheses, and Rationale

The specific objectives of the current study were to evaluate the collagen type and the effects of passage number (freshly isolated and passages 1 and 2) on the proliferation, contraction, and biosynthetic activity of adult canine articular chondrocytes grown in type I and II collagen-glycosaminoglycan matrices. In the light of prior work [47, 48] we hypothesized that there would be differences in the behavior of chondrocytes in type I and II collagen matrices. Based on the prior studies[49], we hypothesized that there would not be substantial differences among the passage numbers, thus supporting the use of passage 2 cells for tissue engineering. The benefit of being able to use passage 2 cells is that more cells would be acquired after two passages.

CHAPTER 2: MATERIALS AND METHODS

2.1 Collagen-GAG Scaffolds

2.1.1 Freeze-Drying

Type I and type II collagen-GAG porous matrices were employed in this study. The type I matrices were fabricated by freeze drying a co-precipitate of type I bovine tendon collagen (Integra Life Sciences, Plainsboro, NJ) and shark chondroitin-6-sulfate (Sigma Chemical, St. Louis, MO)[50]. The matrices have been reported to have a porosity of 87% and a pore diameter of 170 μm (small n)[51]. The type II collagen matrices were fabricated from a porcine derived slurry (Geistlich Biomaterials, Wolhusen, Switzerland) in the same manner as the type I matrices[52]. The type II matrices have been reported to have a porosity of $89 \pm 2\%$ and a pore diameter of $125 \pm 42 \mu\text{m}$ [51]. Appendix A and B detail the protocols for the fabrication of these matrices. For both collagen type matrices, four-millimeter diameter disks were used for this experiment.

2.1.2 Cross-Linking Methods

By cross-linking collagen-GAG matrices, the matrices strength, biocompatibility, resorption rate, and antigenicity can be changed [53]. It has been found by Weadock, *et al.*, that cross-linked collagen has a greater Young's modulus, greater resistance to proteases and a lower degree of swelling than uncross-linked collagen [54]. An advantage of cross-linking matrices is that they become stiffer which allows the matrices to contract less. It has been previously reported [52], that EDAC cross-linked matrices contracted the least as well as having higher rates of DNA content compared to just DHT or UV cross-linked matrices.

Current protocols have shown that the strongest method of cross-linking for collagen-GAG matrices of this nature is 24 hours of dehydrothermal treatment (DHT) followed by 2 hours of EDAC treatment. Therefore, both sets of matrices were initially sterilized and cross-linked by DHT [50]. The extreme dehydration of the DHT treatment cross-links by forming interchain peptidic bonds between amino acid residues [55]. Then 4-mm diameter disks (approximately 2.5 mm thick for type I and 3 mm thick for type II) were cut and additionally cross-linked by carbodiimide treatment (Appendix C) [56]. The matrices were immersed in a carbodiimide solution (EDAC)(14 mM 1-ethyl-3-

(3-dimethylaminopropyl)carbodiimide hydrochloride and 5.5 mM N-hydroxysuccinimide; Sigma) for two hours at room temperature. Through a series of sterile washes, first with phosphate-buffered saline (PBS) followed by two washes with distilled water, excess EDAC was removed from the type I and type II matrices. The EDAC is water-soluble and its unbound and excess chemicals are washed away because it is not incorporated into the amid cross-links that form. The cytotoxic carbodiimide is completely rinsed from the scaffold and collagen-collagen and collagen-GAG cross-links are formed.

2.2 Chondrocyte Isolation from Cartilage and Monolayer Culture

Chondrocytes were isolated from the trochleae of adult canine knees from eight adult mongrel dogs. The cells were obtained using a sequential digestion of pronase (20 U/ml, 1hr) and collagenase (200 U/ml, overnight) as previously described (Appendix D) [57]. Once the chondrocytes were isolated, they were resuspended in culture medium (DMEM/F12, Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS, Hyclone Technologies), 25 µg/ml ascorbic acid (Wako Chemical, Osaka, Japan), and a penicillin/streptomycin/fungizone cocktail (Gibco) and plated in 75-cm² flasks at a 2 million cells/flask. The cells were incubated at 37°C and 5% CO₂. Once cells reached confluence, they were trypsinized, resuspended and replated into 75-cm² flasks. Throughout the study, the chondrocytes from the eight different animals were isolated and cultured separately.

2.3 Cell Seeding of Matrices

The freshly isolated chondrocytes (P0) as well as chondrocytes expanded through 1 (P1) and 2 (P2) passages were seeded into porous type I and type II collagen scaffolds (Appendix E). The matrices were incubated in a cell suspension of 7.5×10^6 cells/ml. Fifteen matrices were incubated in 1 ml of the suspension for 1.5-2 hours on a nutator. By this method approximately 50 % of the chondrocytes attach to the matrices by this dynamic seeding method achieving a cell density of articular cartilage (10,000 cells/mm³)[58]. The matrices were incubated overnight in 0.5 ml complete media per well on agarose-coated wells (24-well plates). The following day, 0.5 ml of complete media was added to each well reaching a volume of 1 ml.

2.4 Culture of Collagen Matrices

Throughout culture of the matrices, media (1 ml) were changed every other day (Appendix E and G). Cultures were terminated at 1-day, 1, 2, 4, 8, and 12 weeks for the type II matrices and at 1-day, 1 and 4 weeks for the type I matrices. For controls, unseeded matrices were cultured as well.

2.5 Chondrocyte-Mediated Contraction

The diameters of cell-seeded and non-seeded matrices were measured 1-day and 1, 2, 4, 8, and 12 weeks post seeding (n=8) for the type II matrices and at 1-day and 1 and 4 weeks post seeding (n=6) for the type I matrices. CMC was determined by subtracting the %contraction of non-seeded constructs from the %change in diameters of cell-seeded constructs.

2.6 Radiolabel Incorporation

At weeks 1,2,4,8, and 12, the P0, P1, and P2 cell-seeded matrices of the type II matrices (n=8) were incubated in medium containing 5 μ Ci/ml each of 3 H-proline and 35 S-sulfate to determine total protein and GAG synthesis respectively (Appendix O). The Type I matrices (n=6) were terminated at 1 and 4 weeks and incubated in the same method as the type II matrices. At the end of the 24-hour radiolabeling period, the matrices were washed (5 x 15 min at 4°C) in PBS supplemented with unlabeled proline (100 mM) and sulfate (500 mM) (Appendix O). The scaffolds were lyophilized and solubilized overnight at 60°C with 1 ml of proteinase K solution (100 μ g in 1 ml 50 mM Tris-HCl buffer with 1 mM CaCl_2). In order to determine the radioactivity content, 100 μ l aliquots of the digest were mixed with 4 ml scintillation fluid (ScintiVerse II; Fisher Scientific Co.) and counted in a liquid scintillation counter (Packard Tri-Carb 4640; Packard Instrument Co.). The 3 H and 35 S counts per minute (cpm) were recorded with corrections for spillover (Appendix P), and then converted to nanomoles of incorporation. Counts were normalized to incorporation time and DNA content (see below).

2.7 Biochemical Assays

2.7.1 Lyophilization and Digestion

Samples were lyophilized (Appendix K) for 24 hours prior to digestion. For digestion, the matrices were solubilized in a proteinase K solution (Appendix L) at a

concentration of 100 μg in 1 ml 50 mM Tris-HCl buffer with 1 mM CaCl_2 . The samples were digested at 60°C overnight with 1 ml of the proteinase K solution.

2.7.2 GAG Analysis

The GAG content of the matrices was determined by the dimethylmethylene blue (DMMB) dye assay[59]. A 100 μl aliquot of the proteinase K digest was mixed with 2 ml of the DMMB dye and the absorbance at 525 nm was measured with a spectrophotometer (Appendix M). The results were obtained by extrapolating from a standard curve using shark chondroitin-6-sulfate. The newly accumulated GAG was determined by subtracting the unseeded values from the sample values.

2.7.3 DNA Analysis

The DNA content of the matrices was measured using the Hoechst 33258 dye method[60]. A 50 μl aliquot of the proteinase K digest mixed with 2 ml of Hoechst dye solution (10% Hoechst dye in 10 mM Tris, 1mM Na_2EDTA and 0.1 M Na CL, pH 7.4) was assayed fluorometrically (Appendix N). The results were extrapolated from a standard curve established using calf thymus DNA. The background fluorescence of the matrix was accounted for by subtracting the values of the unseeded matrices.

2.8 Western Blot Analysis for Type II Collagen

Type II chondrocyte-seeded matrices from P0, P1, and P2 at weeks 1, 4, and 12 were used for Western blot analysis. Samples of the non-cell-seeded type II collagen matrices were also analyzed. The seeded matrices were boiled with sample buffer (1 ml 0.5M Tris at pH 7, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 2-mer-catoethanol, 0.2 ml 1% Bromphenol blue dye) to extract the type II collagen and then loaded into a 7% polyacrylamide gel required for the molecular weight of type II collagen (133 kDa). Type I and type II collagen standards were run along with the molecular weight standard. The gel was run at 90 V for 90 min, then washed in a defixation buffer, and transferred to a nitrocellulose membrane at 100 V for 60 min. After the transfer the membrane was washed in TBS for 5 min and placed in a blocking agent (5% dry milk, Bio-rad) overnight. After blocking, the membrane was incubated with the primary antibody (Iowa Hybridoma Bank, CIICI) at a 1:5 dilution in TBS-T for 2 hours and subsequently washed in a peroxidase-labeled secondary antibody (Sigma, mouse IgG) and a luminol-based

chemiluminescent detection kit (Cell Signal Tech). The complete protocol is detailed in Appendix V.

2.9 Histology

Histology was performed on paraffin embedded cell-seeded and control Type I and Type II CG matrices at each sacrifice point (1-day, 1, 2, 4, 8, and 12 weeks).

2.9.1 Fixation

The specimens of seeded and non-seeded type I and type II CG matrices allocated from histology analysis were fixed in 10 % neutral buffered formalin for 7-10 days.

2.9.2 Embedding and Sectioning

After fixation, the specimens were either dehydrated through a series of alcohol baths by hand (Appendix Q) or by the Tissue Tek automated machine (Appendix R). Samples were then embed in paraffin and were sectioned to a thickness of 7 micrometers.

2.9.3 H & E Staining

Samples were rehydrated, stained with Harris Hematoxylin solution, and counter stained with an Eosin solution. After dehydration, the slides were air-dried and coverslipped with Cytoseal. The nuclei of the cells are evident from a purple stain. The detailed protocol is outlined in Appendix S.

2.9.4 Safrinin-O Staining

Samples were rehydrated, stained with Safranin-O solution, and counter stained with a Fast-Green solution. After dehydration, the slides were air-dried and coverslipped with Cytoseal. The glycosaminoglycans stain red/pink and collagen stains green. The detailed protocol is outlined in Appendix T.

2.10 Statistical Analysis

Data from all eight animals were pooled and are reported as the mean \pm standard error of the mean (SEM). One-factor ANOVA, two-factor ANOVA, three-factor ANOVA, and Fisher's PLSD (protected least squares differences) post-hoc testing were performed using StatView (SAS Institute Inc, Cary, NC). The wide spread of data in several groups prompted the identification of outliers using "box plot" analysis (Statview). This method identified data points above the 90th percentile and below the 10th percentile, which were excluded in additional ANOVAs.

CH. 3: RESULTS

3.1 Matrix Contraction

The chondrocyte-mediated contraction was less than 10 % for the PO, P1, and P2 type II seeded matrices over the 12-week period. The type I seeded matrices with P0, P1, and P2 cells exhibited a slighter greater contraction than the type II matrices. The CMC for the type I matrices at 4 weeks reached approximately 15% for all three passages (Figure 3.1). These results were expected for cross-linked matrices, as previously reported by [56].

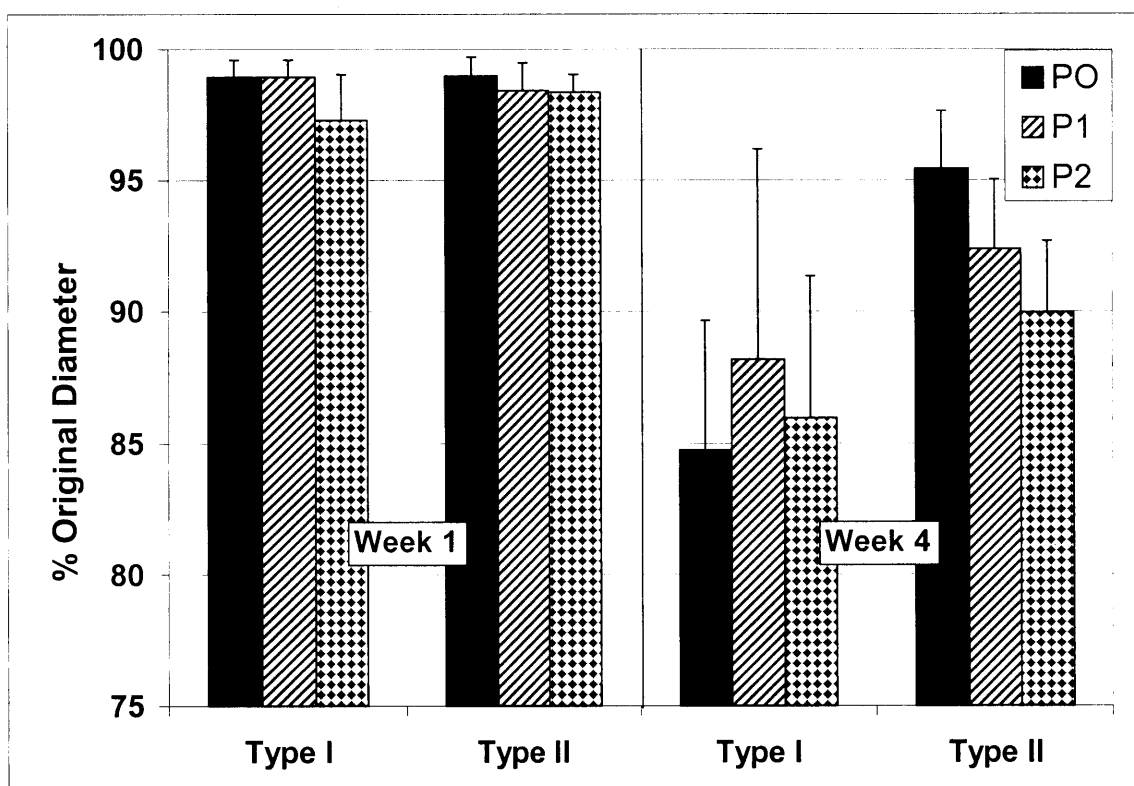


Figure 3.1. Cell-mediated contraction of type I and type II CG matrices. Error bars represent standard error of the mean and n = 6-8 for each passage number per time point.

3.2 DNA Analysis

3.2.1 Type II Seeded Matrices

P0, P1, and P2 cells seeded in the type II matrices continued to proliferate over a 4-week period with 5-, 2.5- and 2.3-fold increases for P2, P1, and P0 respectively, compared to the 1-week value. No significant effect was observed between passages at 1-day, but throughout weeks 1 through 4, cells proliferated at a greater rate with increasing passage number, although, after 4 weeks the P0 and P1 cells continued to increase in number and the P2 cells decreased (Figure 3.2).

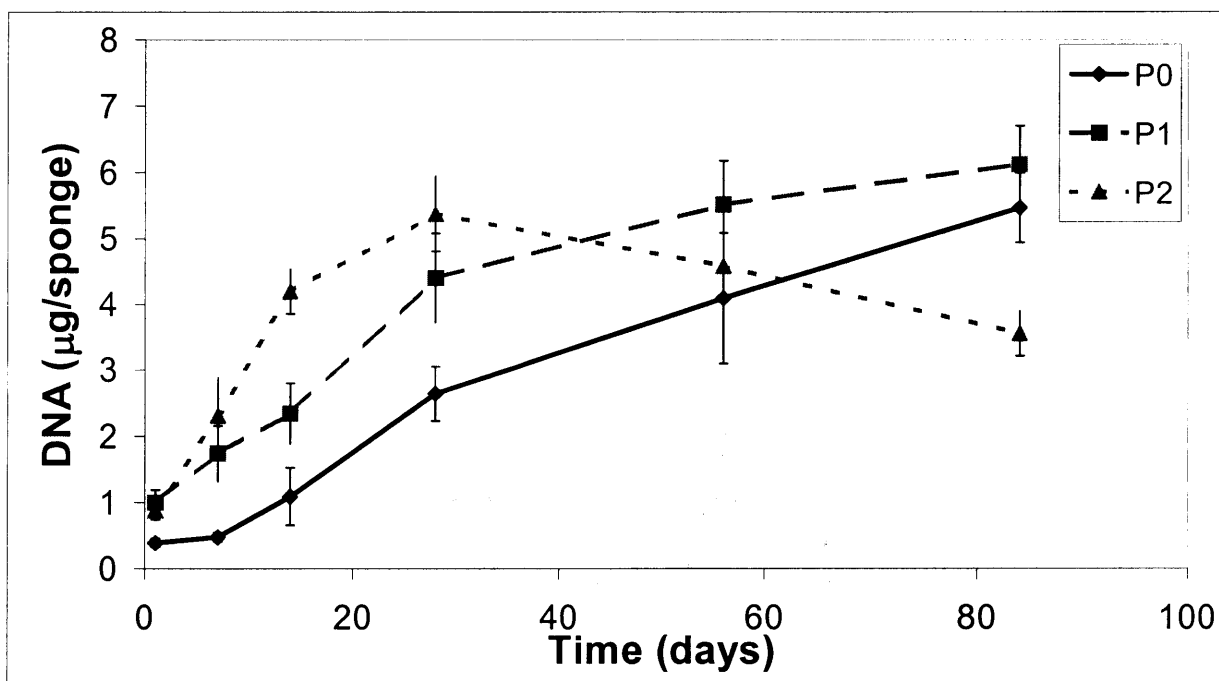


Figure 3.2. DNA content of seeded type II CG matrices. Total DNA content of proteinase K digests of seeded type II CG matrices minus DNA content of non-seeded matrices to account for background fluorescence. Error bars represent standard error of the mean and $n = 6-8$ for each passage number per time point.

Two-factor ANOVA revealed a significant effect of time ($p < 0.001$; power = 1.00) and passage number ($p < 0.005$; power = 0.87). In particular, the significant effect was observed for weeks 1 and 4, weeks 1 and 8, weeks 1 and 12, weeks 2 and 4, weeks 2 and 8, and weeks 2 and 12, and for passage numbers P0 and P1 (Fisher PLSD post-hoc test, $p < 0.02$) and P0 and P2 (Fisher PLSD post-hoc test, $p < 0.02$). Two-factor ANOVA was

also performed for data from 1 to 4 weeks, and a significant effect was observed between all passage numbers and time points being 1, 2, and 4 weeks.

3.2.2 Comparison of Type I and Type II Seeded Matrices

The type I matrices seeded with P0, P1, and P2 chondrocytes continued to

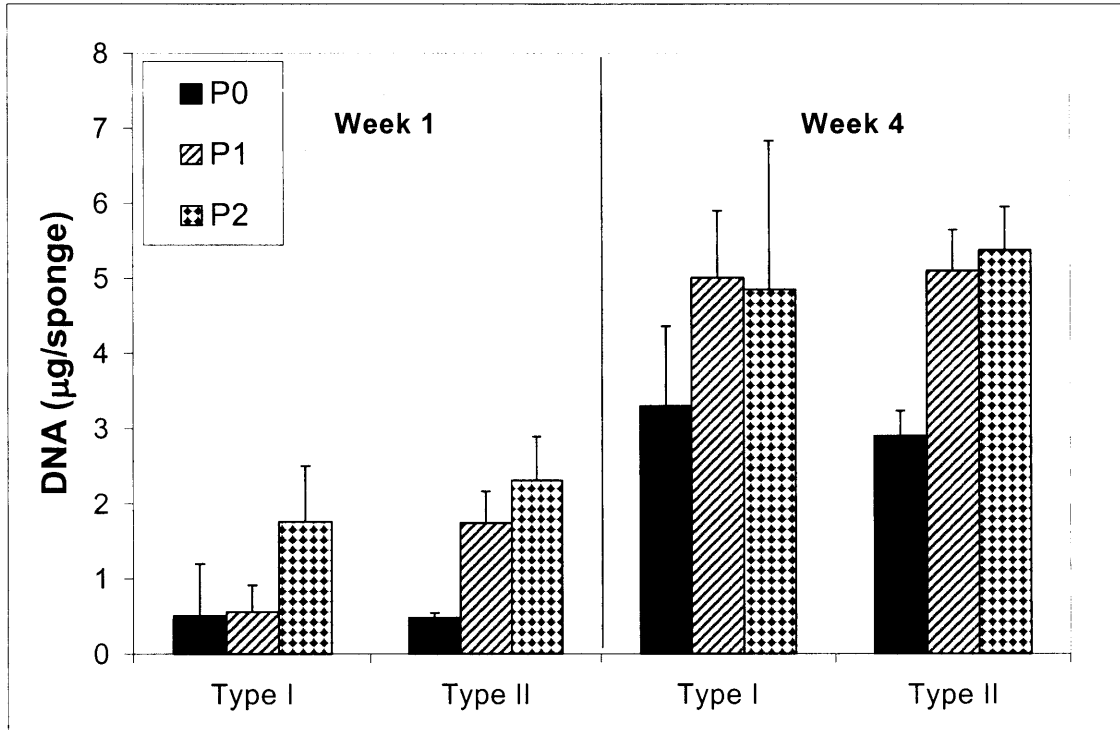


Figure 3.3. DNA content of seeded type I and type II CG matrices. Total DNA content of proteinase K digests of seeded type I and type II CG matrices minus DNA content of non-seeded matrices to account for background fluorescence. Error bars represent standard error of the mean and $n = 6-8$ for each passage number per time point.

proliferate over a 4-week period. The number of cells adherent to the different collagen type matrices after 1-day, reflected in DNA content were comparable (by ANOVA).

Three-factor ANOVA revealed no significant effect for collagen type, but indicated a significant effect of time ($p < 0.0001$; power = 1.0) and passage number ($p < 0.001$; power = 0.98) in regards to DNA content (Figure 3.3). Excluding outliers, one-factor ANOVA revealed a significant effect of collagen type ($p < 0.04$; power = 0.62) at P1, week 1.

3.3 GAG Analysis

3.3.1 Type II Seeded Matrices

The type II seeded matrices demonstrated an increase in the accumulation of GAG for the whole 12-week period for P0, P1, and P2 cells (Figure 3.4). Two-factor ANOVA indicated a significant effect of time ($p < 0.001$; power = 0.99) as well as passage number ($p < 0.02$; power = 0.76). For time, a significant effect was observed between weeks 1 and 4, weeks 1 and 8, weeks 1 and 12, weeks 2 and 4, weeks 2 and 8, and weeks 2 and 12, and for passage number, a significant effect was indicated between P0 and P1 as well as P0 and P2.

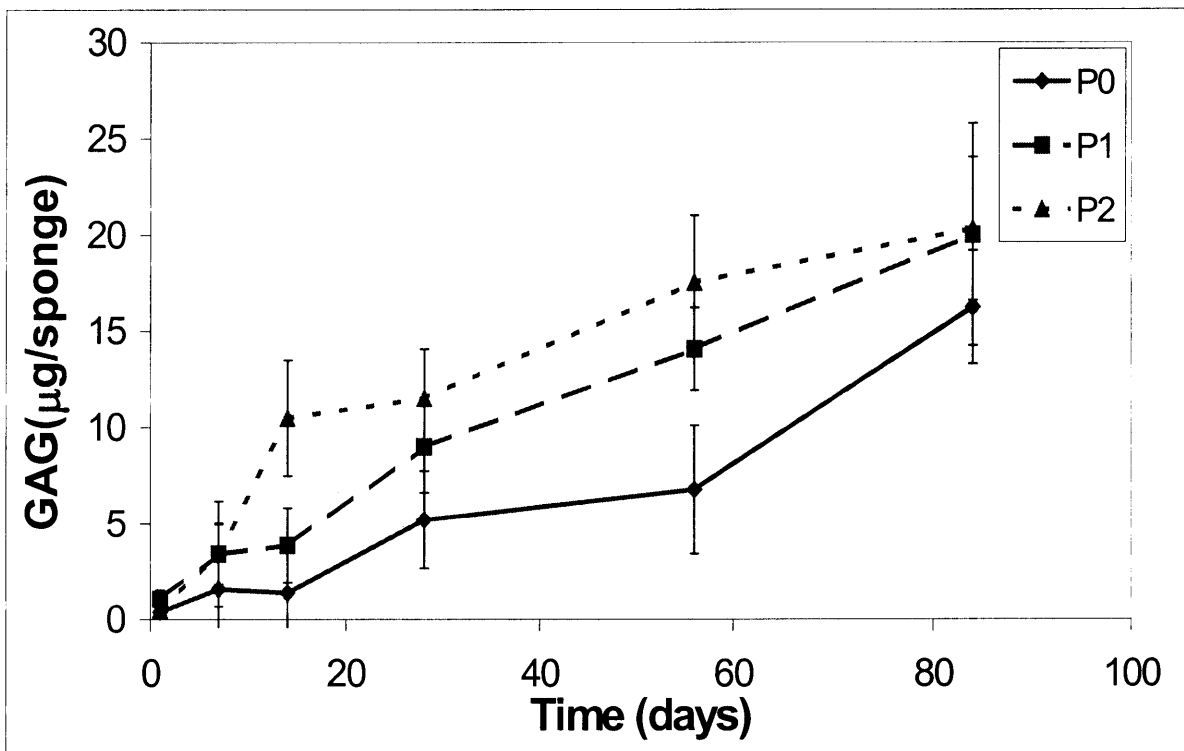


Figure 3.4. GAG content of seeded type II CG matrices. Net GAG accumulation (total GAG minus GAG originally in non-seeded matrices) of proteinase K digests of seeded type II CG matrices. Error bars represent standard error of the mean and $n = 6-8$ for each passage number per time point.

3.3.2 Comparison between Type II and Type I Seeded Matrices

As for the type I data, GAG content demonstrated an increase over the 4-week period. The number of cells adherent to the different collagen type matrices after 1-day, reflected in GAG content were comparable (by ANOVA). At 1 and 4 weeks, the type II seeded matrices averaged $\sim 1\mu\text{g}$ more per sponge (Figure 3.5).

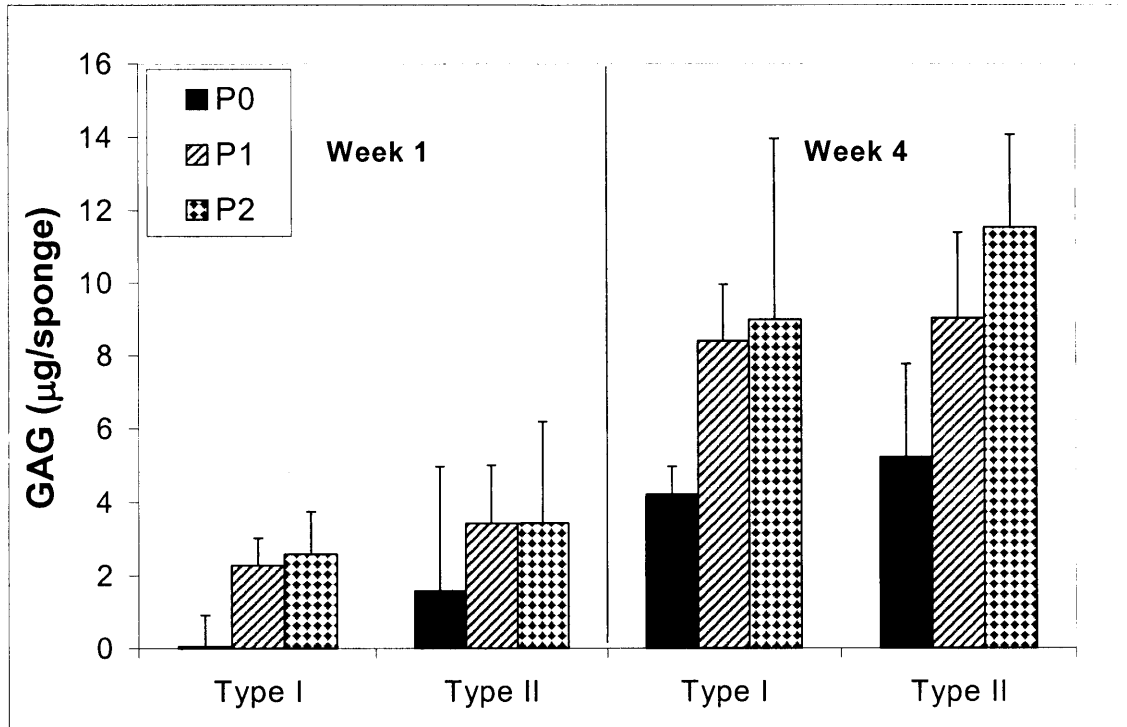


Figure 3.5. GAG content of seeded type I and type II CG matrices. Net GAG accumulation (total GAG minus GAG originally in non-seeded matrices) of proteinase K digests of seeded type I and type II CG matrices. Error bars represent standard error of the mean and $n = 6-8$ for each passage number per time point.

Three-factor ANOVA revealed a significant effect of time between weeks 1 and 4 ($p < 0.003$; power = .90) and a significant effect of passage number, specifically between P0 and P1 (Fisher PLSD post-hoc test, $p < 0.05$) and P0 and P2 (Fisher PLSD post-hoc test, $p < 0.03$). For the time periods of week 1 and week 4, three-factor ANOVA indicated no significant effect for collagen type.

3.4 Radiolabel Incorporation

3.4.1 Type II Seeded Matrices

The type II-seeded matrices exhibited biosynthetic activity throughout all 12 weeks of the study. Although activity was evident throughout the whole 12 weeks, a gradual decrease in biosynthetic activity occurred with time. Except for a few time points, there were no remarkable differences among the passage numbers.

Of interest was the low biosynthetic activity of the P0 cells at 1 week. Two-factor ANOVA revealed no significant effect of passage number, but a significant effect of time between weeks 1 and 4 (Fisher PLSD post-hoc test, $p < 0.04$) as well as weeks 1 and 12 (Fisher PLSD post-hoc test, $p < 0.008$) for ^3H -proline incorporation (Figure 3.6a). One-factor ANOVA indicated that at week 1, a significant effect existed for passage number ($p < 0.002$), while no other effects were noted at weeks 2, 4, 8, and 12.

As for ^{35}S -sulfate incorporation, two-factor ANOVA revealed no significant effect for passage number, but significance for time ($p < 0.05$), specifically between weeks 1 and 2, weeks 1 and 4, weeks 1 and 8, weeks 1 and 12, and weeks 2 and 12 (Figure 3.6b). Where one-factor ANOVA demonstrated no significance between passage numbers for any of the selected time intervals.

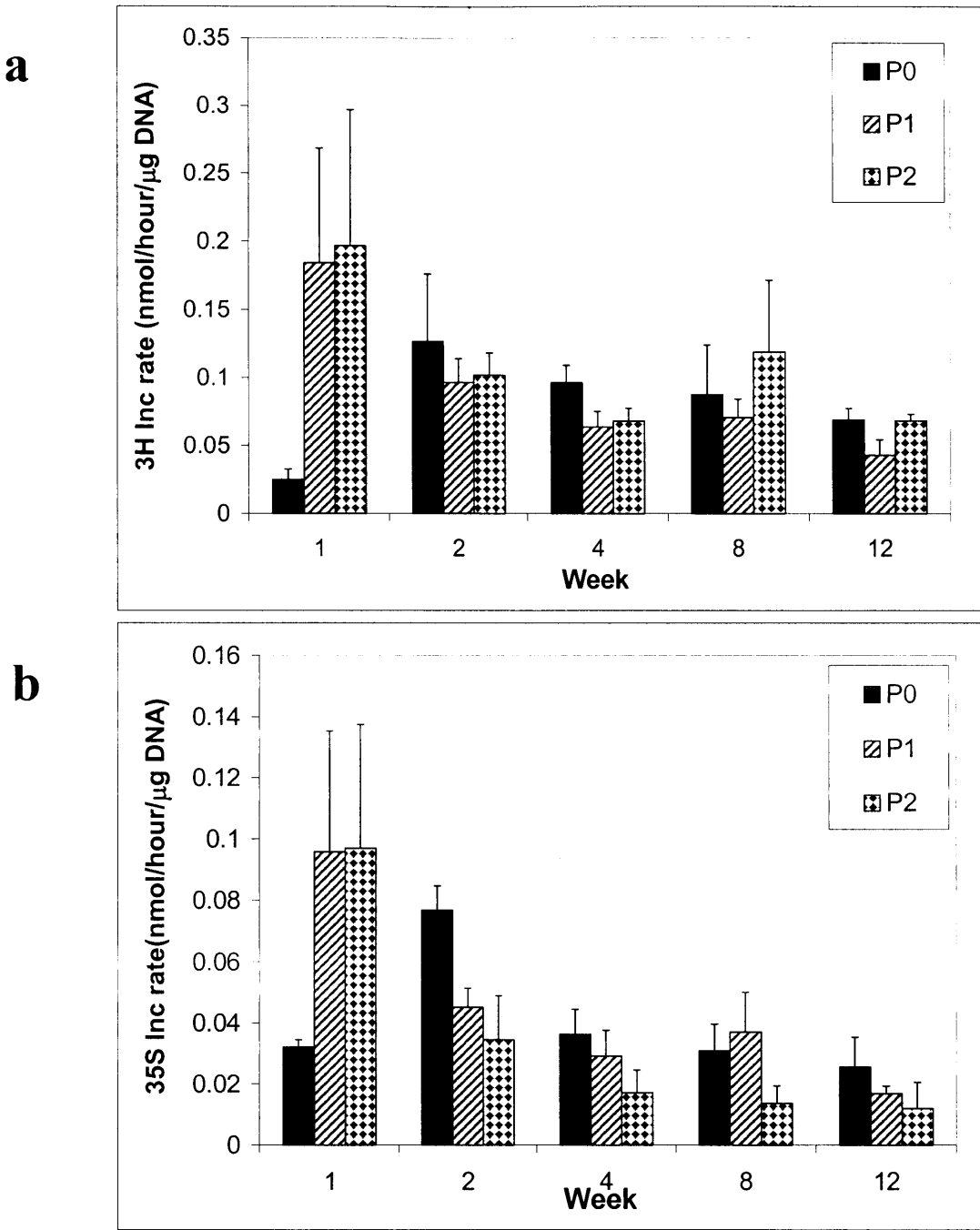


Figure 3.6. Protein and GAG Biosynthesis of type II CG matrices. Accumulation of newly synthesized a) total protein measured by ^3H -proline and b) GAG measure by ^{35}S -sulfate incorporation for type II CG matrices. Amount of radiolabel incorporated into the matrices was normalized to time in culture and DNA content. Error bars represent standard error of the mean and $n = 6-8$ for each passage number per time point.

3.4.2 Comparison between Type II and Type I Seeded Matrices

Biosynthetic activity for both type I and type II CG seeded matrices was observed through a 4-week period (Figure 3.7), although there was a decline. Of interest was the generally lower biosynthetic activity of the P0 cells. Three-factor ANOVA revealed a significant effect of time in culture ($p < 0.0001$) and passage number, specifically between P0 and P1 (Fisher PLSD, $p < 0.05$), but no significant effect of collagen type on protein synthesis (Figure 3.7a). Three-factor ANOVA revealed no significant effect of time, passage, or collagen type on GAG synthesis (Figure 3.7b). At 1-week the protein and GAG synthesis by the P1 and P2 cells in the type II matrices were 1.5- to more than 2-fold greater than in the type I matrix, but owing to the variation in the data these differences were not statistically significant (Figures 3.7a and b). By 4 weeks there were no notable differences in the protein and GAG synthesis among groups (Figures 3.7a and b). For all groups, except the 1-week GAG synthesis values for P1 and P2 cells seeded in type I matrices, were comparable.

When the outliers were omitted, two-factor ANOVA revealed a significant effect of collagen type on GAG synthesis at week 1 (Fisher PLSD, $p < 0.04$), with higher levels found in the type II collagen matrices. By 4 weeks two-factor ANOVA indicated a significant effect of protein synthesis (Fisher's PLSD, $p < 0.02$). Also, one-factor ANOVA was performed on the data excluding outliers and a significant effect of collagen type on protein synthesis was observed at P0, week 4 ($p < 0.02$; power = 0.74) and P2, week 1 ($p < 0.03$; power = 0.71).

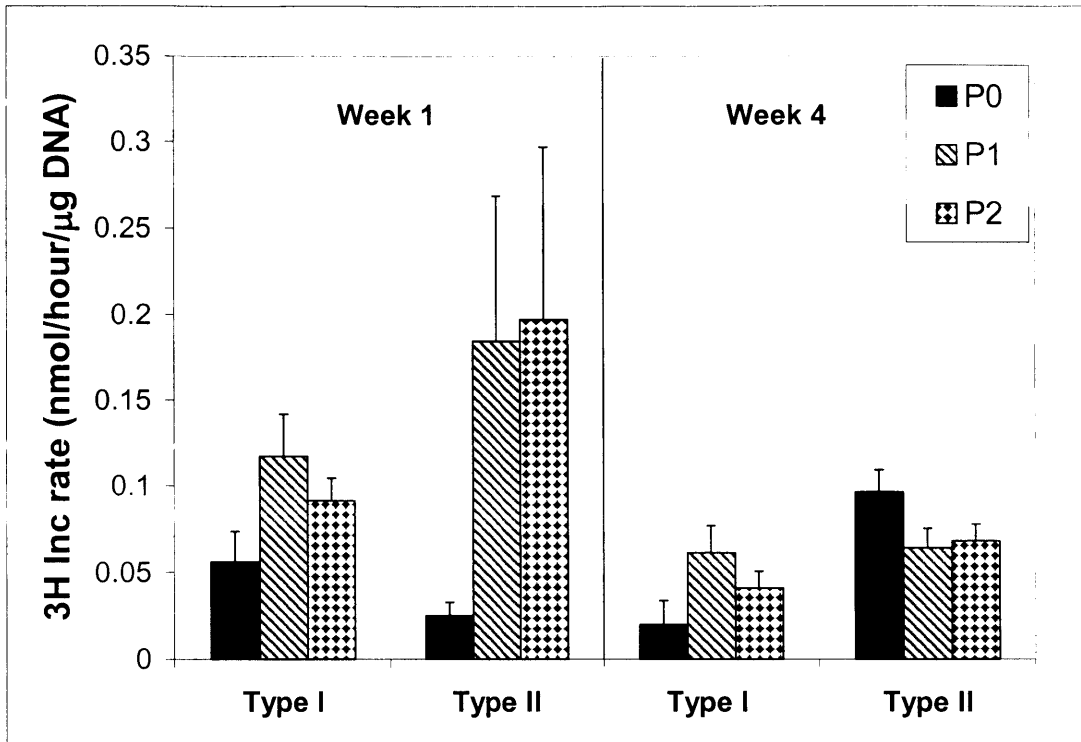
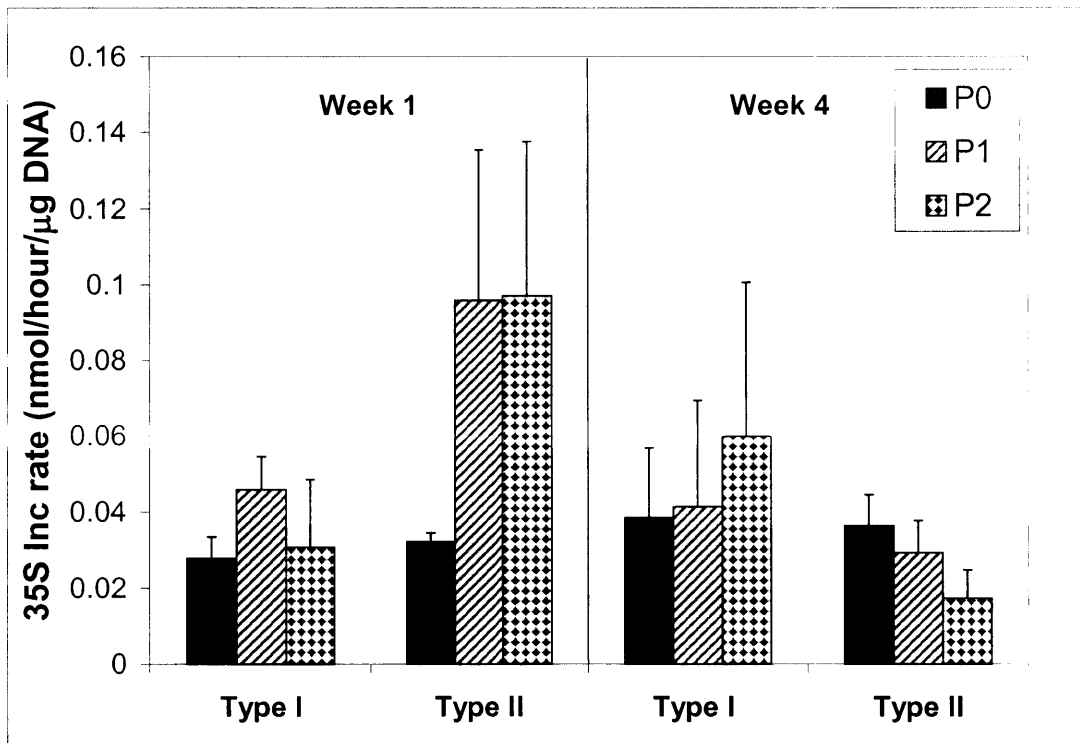
a**b**

Figure 3.7. Protein and GAG Biosynthesis of type I and type II CG matrices. Accumulation of newly synthesized a) total protein measured by ^3H -proline and b) GAG measure by ^{35}S -sulfate incorporation for type I and type II CG matrices. Amount of radiolabel incorporated into the matrices was normalized to time in culture and DNA content. Error bars represent standard error of the mean and $n = 6-8$ for each passage number per time point.

3.5 Western Blot Analysis for Type II Collagen

Western blot films of the non-cell-seeded type II collagen matrix displayed no band for type II collagen (Figure 3.8) due to the effect of the EDAC treatment. This was concluded based on a pilot Western analysis of type II collagen matrices receiving dehydrothermal treatment only, showing the appropriate type II collagen band. This finding indicated that the type II collagen detected by Western blot analysis in the chondrocyte-seeded type II collagen scaffolds used in this study was that produced by the cells.

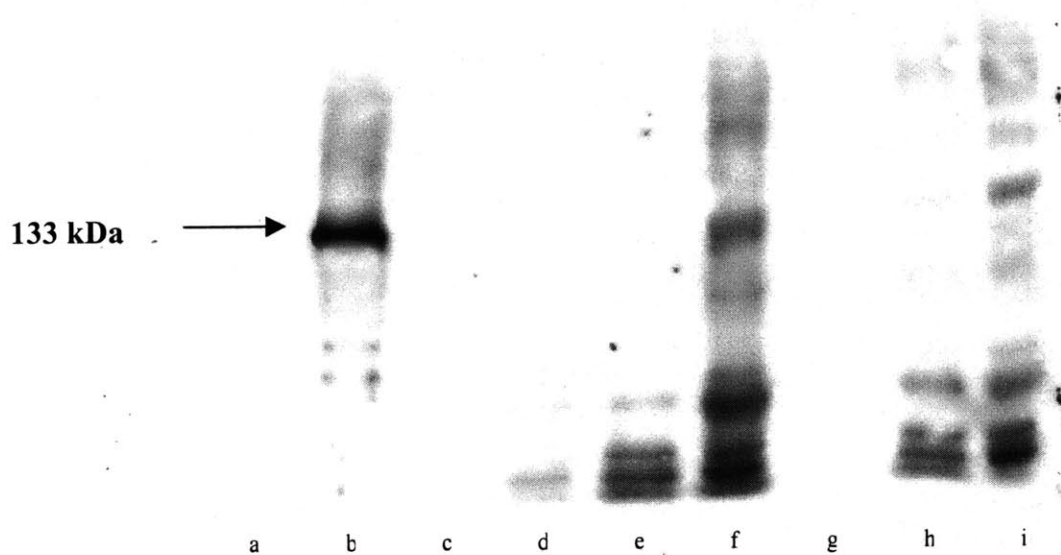


Figure 3.8. Western blot for type II collagen. Western blot reveals presence of type II collagen in the chondrocyte-seeded EDAC cross-linked matrices for P1 cells after 12 weeks and P2 cells after 4 and 12 weeks. a=type I standard; b=type II standard; c=non-seeded type II EDAC cross-linked matrix; d=P1 seeded EDAC cross-linked matrix at 1-week; e= P1 seeded EDAC cross-linked matrix at 4-weeks; f= P1 seeded EDAC cross-linked matrix at 12-weeks; g= P2 seeded EDAC cross-linked matrix at 1-week; h= P2 seeded EDAC cross-linked matrix at 4-weeks; i= P2 seeded EDAC cross-linked matrix at 12-weeks.

The Western blot analysis demonstrated for the type II seeded CG matrices, P1 cells at week 12 and P2 cells at weeks 4 and 12 (Figure 3.8) showed the presence of type II collagen. For the type II study, Western blot analysis of chondrocyte-seeded EDAC matrices displayed that for longer periods of culture and higher passage numbers there is a deposition of type II collagen in the scaffold. It can be reasoned that this is newly synthesized type II collagen because a non-seeded EDAC control matrix displayed no band for type II collagen after being exposed to the primary type II collagen antibody.

3.6 Histology

For the chondrocyte-seeded type II collagen matrices, the P0, P1, and P2 cells were distributed throughout the matrix from 1 week onward with at least a 1-2 cell layer formed on the outside of the matrix. As time progressed, the outside cell layer increased. Safranin-O staining indicated an increase in proteoglycan accumulation with passage number as well as time with later passage and time points displaying larger pink stained portions specifically on the outside of the matrix. The only apparent differences observed between the type I and type II seeded matrices were that the type I matrices appeared to have more elongated cells, less cell distribution throughout the matrix, and a less dense cell layer at the outer edges of the matrix (Figure 3.9).

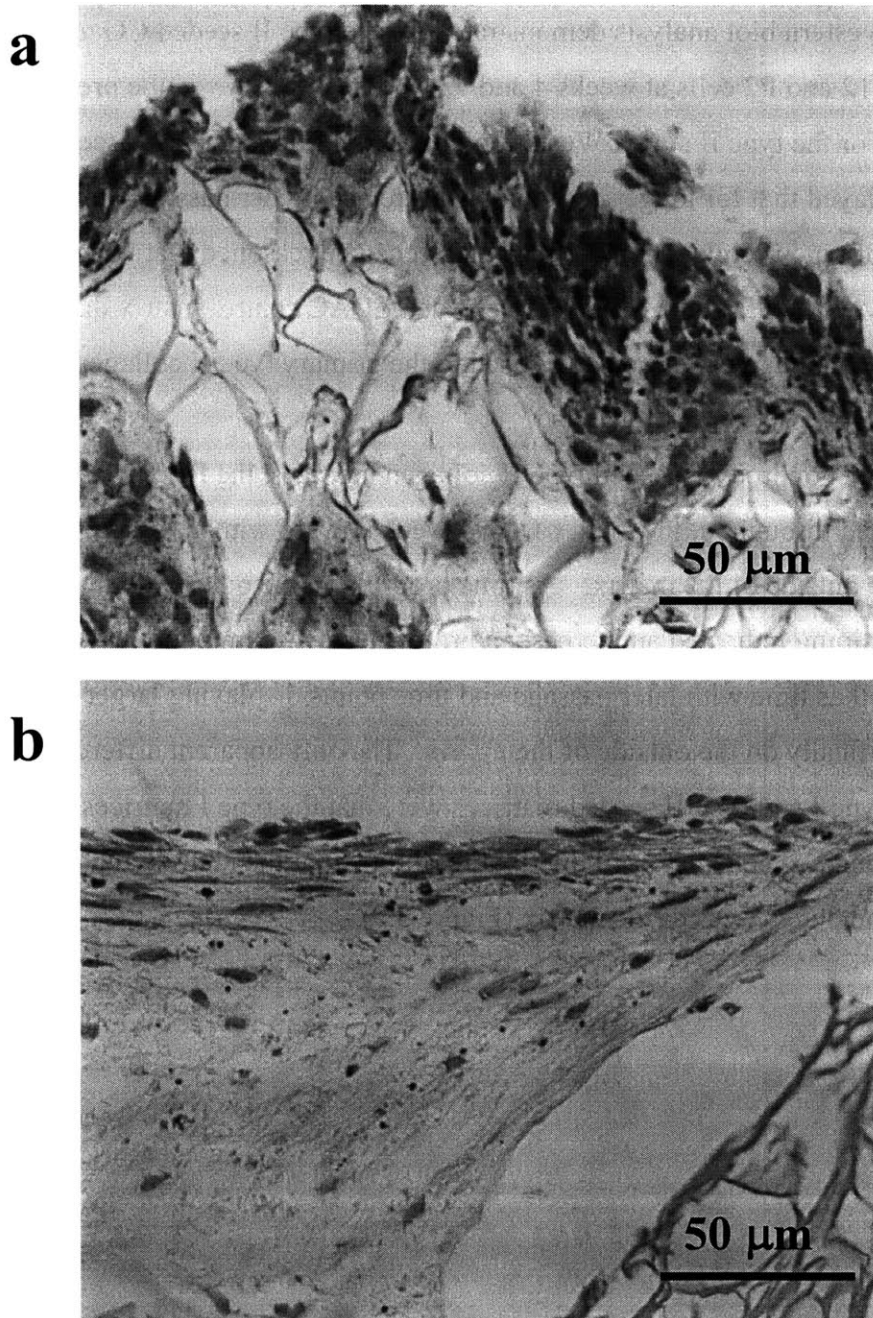


Figure 3.9. Micrographs of type I and type II seeded matrices. H&E staining of type I and type II matrices after 4 weeks in culture with P2 cells at same magnification. a) Type II matrix sample. b) Type I matrix sample

CHAPTER 4: DISCUSSION

A notable finding of this thesis was that the serial subculture of adult canine articular chondrocytes through 2 passages in monolayer did not adversely affect their proliferation or biosynthetic activity when they were subsequently grown in collagen-GAG matrices for 4 weeks, as might be done to prepare cell-seeded constructs as implants for articular cartilage tissue engineering. This supports one of the working hypotheses. Of importance was the finding that the P2 cells, as well as the P1 cells, grown in the type II collagen matrices retained their capability to synthesize type II collagen indicating that they maintained a critical phenotypic trait of articular chondrocytes.

It has been well accepted that during the serial passaging of articular chondrocytes in monolayer culture, required to increase the number of cells for implantation, the cells adopt many of the phenotypic traits of fibroblasts, becoming elongated and synthesizing type I rather than type II collagen. However, in the seminal investigation of this phenomenon [49, 61], it was only after the third passage that type I collagen synthesis was initiated, with the cells continuing to synthesize type II collagen. The rapid decline of the synthesis of type II collagen occurred during the third to fifth passage. Western blot analysis of the seeded type II collagen constructs demonstrated that P1 and P2 cells from 4 to 12 weeks are actively synthesizing type II collagen. This finding also supports prior studies [62, 63] that showed that articular chondrocytes expanded in monolayer culture through 2 passages express the chondrocytic phenotype when grown in selected environments. Collectively these data indicate that adult chondrocytes expanded in monolayer through two passages and grown in a type II collagen matrix have the potential to proliferate for up to four weeks and display rates of biosynthesis comparable with those of freshly isolated cells.

After 4 weeks, in the type II collagen scaffolds, the P2 cells declined in number in the matrices while the P0 and P1 cells continued to proliferate. Of potential importance was the finding in this study that the rate of cell proliferation in the type II matrix increased with passage number up to 4 weeks. This may further commend the use of a P2-chondrocyte-seeded type II collagen matrix for implantation prior to 4 weeks, when cell proliferation could be expected to increase more than might be found with freshly

isolated or P1 cells. That the rate of synthesis of protein and GAG after 4 weeks, and the amount of newly synthesized GAG accumulated in the scaffolds, was also greater for the P2 cells when compared to the freshly isolated chondrocytes further commends this tissue engineering approach. Once the cell-seeded construct is implanted (*e.g.*, after 4 weeks in culture) it is unlikely that the *in vitro* findings from this study would predict the *in vivo* behavior because of the effects of the myriad of endogenous growth factors and other agents affecting cell behavior.

This study also sought to test the hypothesis that there would be differences in the behavior of the chondrocytes in the type I and II matrices. Biochemical composition of tissue engineered scaffolds to a considerable degree, determine the behavior of seeded cells [30, 31, 48]. For the generation of articular cartilage, the chondrocytic phenotype must be upheld. When grown in monolayer, chondrocytes dedifferentiate, acquire a fibroblast-like morphology, and synthesize type I collagen rather than type II collagen [64, 65]. In this study, type I collagen and type II collagen cross-linked matrices were employed because in order to maintain the phenotype of the chondrocyte, a three-dimensional matrix is required [42, 64, 66]. The re-expression of the chondrocyte phenotype lost during monolayer passaging has been shown to occur when the cells are subsequently introduced into certain culture conditions (*e.g.*, agarose gels) [62, 63]. These matrices were analyzed for proliferative, contractile, and biosynthetic behavior.

The initial adherence of the cells to both types of matrices was comparable. As reflected by DNA content, for this study, chondrocytes, either freshly isolated or expanded in monolayer were able to infiltrate both porous matrices of either type I or type II collagen. One-factor ANOVA for the day-1 results demonstrated that collagen type (type I or type II) did not affect the amount of cells adhering to the porous matrix. This possibly could indicate that for initial seeding, composition (type I or type II collagen) of matrix is less important to porosity and pore diameter of the matrix being that the two matrices employed in this study were similar in that manner[51]. Assuming a 50% seeding efficiency[52], either composition of matrix (type I or type II) results in an initial cell density of 10,000 cell/mm³ which is similar to that of adult human articular cartilage[58].

Cells continued to proliferate up to 4 weeks in both collagen type matrices, and synthesized protein and GAG through 4 weeks of growth. Even though continued biosynthesis occurred, there was a general decrease as time in culture increased. These results are consistent with other studies that also reported a decrease in biosynthetic activity with culture time for newborn bovine chondrocytes in agarose [67] and peptide gels [68], and adolescent bovine chondrocytes in porous collagen-GAG constructs [69]. For the matrices explored in this study, the rates of proline incorporation (0.02-0.2 nmol/ μ g DNA/hour) were double to that of sulfate incorporation (0.01-0.1 nmol/ μ g DNA/hour). The higher levels of proline incorporation in comparison to sulfate incorporation may be a consequence of the chondrocytes, be it freshly isolated or passaged synthesizing more proteins than proteoglycans.

Another factor to consider in analyzing the rates of biosynthesis is that the biosynthetic activity recorded was of that of the scaffold itself. It has been reported by Lee [52], that for type II CG matrices (same composition as employed in this study), that approximately 70 % of the newly synthesized macromolecules over a 24-hour period were released into the medium. It was reported by Lee, that as culture time increased there was a decrease in the amount of newly synthesized macromolecules released into the medium, but these values were still relatively high in comparison to other studies that reported less than 2% of newly synthesized glycosaminoglycans produced in cartilage explants [70] or in agarose gels [67] were released into the medium. Since the objective of this study was to explore the behavior of the seeded-collagen-GAG matrix itself, only the biosynthesis of the macromolecules adherent to the matrices was analyzed. It is possible, that the cells when implanted in either the type I or type II matrix have even greater differences in biosynthetic activity as stimulated by collagen type even though statistical evidence of the adherent macromolecules synthesized does not support a difference.

With respect to DNA content, GAG content, and biosynthetic activity the seeded type II collagen matrices had greater values in comparison to the seeded type I collagen matrices. These increased values could be a sign of the type II collagen stimulating the chondrocytes proliferation and proteoglycan synthesis[31]. Although these increased values were observed, statistical analysis indicated no significance for those cell

behaviors in either collagen type matrices. When outliers were omitted, a statistical difference was observed in favor of the type II matrices. The variation in the data could be attributed to the cells coming from eight different canines.

Although, DNA content, GAG content and GAG biosynthesis were not statistically effected by collagen type, it is worth noting that the type II collagen matrix had higher rates of protein synthesis than the type I collagen matrix. Due to the reports of previous studies[31, 43, 48], it could be said that a type II collagen matrix would be more suitable to pursue for tissue engineering of articular cartilage. Using collagen-glycosaminoglycan matrices, a prior investigation found that while the majority of the cultured chondrocytes seeded in the type I collagen matrices had a fibroblastic morphology, the majority of the cells in the type II collagen matrices had a chondrocyte morphology and displayed an increase in GAG production, indicating, perhaps, that the cells are better able to redifferentiate in the type II collagen scaffolds [71]. These findings were supported by other investigations which demonstrated differences in the response of chondrocytes, grown on substrates comprising type I or II collagen, to selected growth factors [72, 73]. It has been reported that a type II matrix facilitates the spherical, chondrocytic cell shape for the majority of cells in comparison to the majority of chondrocytes in a type I matrix retain an elongated and flattened morphology similar to that of a fibroblast[48]. Pieper, *et al.*, observed that chondrocytes in type II collagen based matrices had a distribution of cells throughout the matrix where the majority of chondrocytes in the type I collagen matrices were located at the margins [74]. Also other studies have found that chemical composition, in particular hydrogels, allows for the chondrocytes to maintain there spherical morphology associated with the synthesis of type II collagen[42, 64].

As expected, the EDAC cross-linking protocol employed in this study for both the type I and II collagen scaffolds imparted a sufficient degree of mechanical stiffness to resist the amount of chondrocyte-mediated contraction reported for collagen matrices with less cross-linking [75]. Prior work has demonstrated that this cell-mediated contraction is associated with the expression of SMA [76]. That the type I matrices underwent a slightly greater degree of contraction than the type II scaffolds over a 4-

week period may be related to the fact that the EDAC cross-linking protocol resulted in a greater increase in the stiffness of the type II matrix compared to the type I material.

Potential advantages of a type II collagen matrix relative to a scaffold fabricated from type I collagen have been noted previously. It has been reported that a type II matrix facilitates the spherical, chondrocytic cell shape for the majority of cells in comparison to the majority of chondrocytes in a type I matrix that adopt an elongated and flattened morphology similar to that of a fibroblast [77]. The prior work also demonstrated increased chondrocyte proliferation and proteoglycan synthesis in type II collagen scaffolds [77].

The results of this study serve as the foundation for future investigations of the preparation of chondrocyte-seeded type II constructs as implants for cartilage regeneration *in vivo*.

CHAPTER 5: CONCLUSIONS

The objective of this thesis was to develop a better understanding of chondrocytic behavior in type I and type II collagen-glycosaminoglycan for tissue engineering of articular cartilage. The results reported support the hypothesis that there were no substantial differences between passage numbers and that passage 2 cells can be employed for future tissue engineering studies. On the other hand, in regards to cell proliferation, contractile behavior, and biosynthetic activity of chondrocytes, matrix composition (collagen type) did not have a significant effect on the behavior of the seeded chondrocytes. An important factor to note was that the chondrocytes seeded in the type II matrices were actively synthesizing type II collagen up to 12 weeks in culture. Since prior studies comparing type I vs. type II collagen matrices demonstrated that a much greater percentage of chondrocytes maintained their phenotype when seeded in a type II collagen matrices, it would be worthwhile to explore a type II collagen matrix as a potential implant for tissue engineering.

Using the data recorded in this study, future studies may focus on other variables such as growth factors and serum-free media on the proliferative and biosynthetic activity of P2 cells seeded in type I and type II matrices. Ultimately, the findings in this study will aid in the enhancement of a tissue-engineered implant for articular cartilage defects.

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APPENDIX A. TYPE I COLLAGEN GLYCOSAMINOGLYCAN

Protocol from Fiber & Polymers Lab, for skin regeneration template.

Slurry

- 1) Cool blender for 30 minutes to 4°C (according to directions for cooling blenders on cooling bath next to blenders).
- 2) Fill blender with 600 ml of 0.05 M acetic acid (2.9 ml glacial acetic acid + distilled H₂O to 1L). The glacial acid is located in the cabinet across from the blenders labeled "acids."
- 3) Weigh 3.6 g of dry bovine tendon collagen (Integra Life Sciences)
- 4) Place collagen in blender and blend on high for 90 minutes.
- 5) Dissolve 0.32 g chondroitin-6-sulfate (c-6-s) in 120 ml of 0.05 acetic acid using a magnetic stirrer (do this while blending the collagen to assist dissolving the c-6-s).
- 6) Add c-6-s solution to blender over 15 minutes using peristaltic pump (continue blending).
- 7) Blend for an additional 90 minutes on high.
- 8) If using slurry immediately, transfer to a vacuum flask and de-gas for 10-30 minutes.
- 9) If using later, pour out slurry and refrigerate. When ready to use, blend slurry for 15 minutes at 4°C and then de-gas as above.

Vitreous Freeze-drying

- 1) Turn on VirTis freeze-drier.
- 2) Make sure to drain condenser (tube under condenser) and turn on condenser.
- 3) After condenser has cooled, turn on freeze.
- 4) Wait for shelf to cool down to -45°C (may only reach -40°C), at least 1 hour. (The new protocol puts the tray on the shelf while it reaches -45°C).
- 5) Clean the freeze-drying tray with 0.05 M acetic acid.

- 6) Pipette slurry into freeze-drying trays - usual tray is divided into 3 sections - ~220 ml/section yields foam thickness of ~3.5-4 mm; ~180 ml/section yields foam thickness of ~2.5 mm. (for future use, new smaller trays have been purchased and amounts of slurry have been scaled down - check protocol at Fiber and Polymer's Lab).
- 7) Make sure there are no air bubbles in slurry. If there are bubbles, remove them with a syringe.
- 8) Place tray of slurry into freeze drier and freeze (~1.5 hours).
- 9) When slurry has frozen, turn on vacuum (condenser must be at -50°C before vacuum is turned on) and press door shut to make sure it seals (also, make sure chamber release is off; do not leave until you are sure the door is sealed and the pump is pulling a vacuum).
- 10) Pull vacuum at -45°C until it is below 200 mtorr (~1-2 hours).
- 11) Once vacuum is below 200 mtorr, set temperature to 0°C and turn on heat and leave on freeze button.
- 12) Sublimate overnight (at least 12 hours).
- 13) In the morning, set temperature to 20°C and turn off freeze button.
- 14) When temperature reaches 20°C (~30 minutes), turn off heat, vacuum, and condenser buttons. Turn on chamber release.
- 15) After vacuum is released, turn off VirTis, remove tray, and open drain plug on condenser.

DHT Cross-linking

After freeze-drying, the thickness of the matrix should be measured with a micrometer. Then, the matrix should be placed into the DHT oven for dehydrothermal cross-linking for 24 hours. The conditions of the vacuum oven are 1 atm and 105° C. The matrix sheets are placed in aluminum foil with one end open. After 24 hours, the matrix should be stored in a dessicator prior to use. The instructions for starting the vacuum on the DHT and purging the oven are on it.

Storage

Unless hydrated, all matrix should be stored in a dessicator with blue desiccant.

APPENDIX B. TYPE II COLALGEN-GLYCOSAMINOGLYCAN

Based on protocol developed by Lee. Using Chondrocell slurry from Geistlich Biomaterials. Slurry stored at 4°C.

- 1) Transfer more than desired amount of slurry to 50 ml centrifuge tube.
- 2) Centrifuge for 5 minutes at 3500 rpm to de-gas slurry.
- 3) Pipette slurry into 6-well plates wells (A new protocol may be developed using the smaller metal trays in the Fibers and Polymers Lab). 3.5 ml slurry/well is the standard volume for "cartilage protocol" yielding matrices ~2-2.5 mm thick. Pipette reading is not actual volume due to slurry sticking to inside of pipette. Pipette slurry into center of well and tap/bang plate on countertop to evenly distribute slurry, remove bubbles with 1 cc syringe and needle.
- 4) Freeze-dry (freeze with lids on, remove lids before pulling vacuum) as for type I CG slurry (see Appendix A).
- 5) Follow with DHT cross-linking (Appendix A).

APPENDIX C. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) CROSS-LINKING

Modified from Lee. Based on Pieper et al.

Materials

EDAC (Sigma #E-7750; store desiccated in the freezer): MW = 191.7 g/mol

N-hydroxysuccinimide (NHS) (Sigma #H-7377; store desiccated): MW = 116 g/mol

Methods

6 mmol EDAC/g collagen

5:2 ratio EDAC:NHS

For example: If using 3-4 mm thick 9 mm-diameter CG discs with an estimated mass of 0.005 g each, for 48 discs use 100 mL of 1.44 mM EDAC (0.276 g) and 0.56 mM NHS (0.064 g).

- 1) Take out EDAC and let warm up one hour prior to use (so that moisture doesn't condense inside bottle when opened – EDAC is moisture sensitive).
- 2) Weigh out amounts of EDAC and NHS. For 100 mL final solution, use 0.276 g EDAC and 0.064 g NHS.
- 3) Hydrate matrices in half the final volume (i.e.: for 100 ml final volume, hydrate in 50 ml) of sterile, dH₂O.
- 4) Dissolve EDAC and NHS in half the volume of dH₂O water (i.e.: for 100 ml final volume, dissolve in 50 ml; make up fresh for each use). Swish gently until dissolved (few seconds). Do not stir solution.
- 5) Sterile filter (0.45 μm) into sterile container (or directly into container with hydrated matrices).
- 6) With combined solution cross-link at room temperature for 2 hours in 50 mL centrifuge tubes on a rocker.
- 7) Rinse in sterile PBS. Change to fresh, sterile PBS and place on rocker for another 2 hours to remove residual uncross-linked EDAC.
- 8) Rinse 2x10 minutes in sterile dH₂O.
- 9) Store at 4°C for up to one week before use in sterile dH₂O (effects of longer storage unknown). In practice no one has used EDAC cross-linked matrix more than 2 days after cross-linking.

APPENDIX D. HARVESTING ARTICULAR CHONDROCYTES

OR Supplies

For removing whole joint:

- Scalpel handle (#3)
- Scalpel blades (#10)
- Sterile forceps
- Sterile mosquitos
- Sterile gloves
- Coping saw
- Sterile fields
- Sterile PBS to moisten towels

Lab Supplies

- Sterile spatula
- Sterile forceps
- Scalpel handle (#3)
- Scalpel blades (#10)
- Sterile 50 ml centrifuge tube per 2 joints filled with ~30 ml complete PBS
- Complete PBS (0.1 ml Pen/Strep/Fungizone cocktail (100X; Gibco #15240-062) per 10 ml D-PBS (Gibco #14190-144)
- Clean stir plate in incubator (one that does not heat up too much during prolonged operation)

Solutions

Pronase solution (20 U/ml) - approx 40 ml/2 joints

Dissolve appropriate amount of pronase (Sigma protease type XIV #P 5147) in 10 ml DMEM/F12 (Gibco #11320-033)

$$\text{pronase}(mg) = \frac{V \times 20U / ml}{\#Units / mg} \quad \text{where } V = \text{volume of solution}$$

Sterile filter (0.2 μ m) solution (use syringe filter; it will take time for pronase to dissolve and it will be difficult to pass solution through filter)

Add remaining volume of DMEM/F12 (ex: 30 ml if making 40 ml/2 joints)

Add appropriate amount of 100X pen/strep/fungizone cocktail (ex: 0.4 ml)

Collagenase solution (200 U/ml) - approx 40 ml/2 joints

Dissolve appropriate amount of collagenase (Worthington Biochemical CLS2) in 10 ml DMEM/F12 (this dissolves quickly)

$$\text{collagenase}(mg) = \frac{V \times 200U / ml}{\#Units / mg} \quad \text{where } V = \text{volume of solution}$$

Sterile filter (0.2 μ m) solution (again, use syringe filter and it should be much easier to filter than pronase solution)

Add remaining volume DMEM/F12

Add appropriate amount of 100X pen/strep/fungizone cocktail

Complete media

1 bottle DMEM/F12 (~530 ml: Gibco #11320-033)

60 ml Fetal Bovine Serum (Hyclone SV 3002.01)

6 ml 100X Penicillin/Streptomycin/Fungizone cocktail (Gibco #15420-062)

6 ml 100X ascorbic acid solution (final concentration 25 μ g/ml)

100X solution is 0.25 g ascorbic acid (Wako Chemical #D13-12061) in 100 ml DMEM/F12; sterile filtered, store frozen

Methods

- 1) Using scalpel blades to remove slices of articular cartilage from joint surfaces and place in complete PBS.
 - a. Don't dig into the hard calcified cartilage or subchondral bone, cartilage should offer little resistance to the blade; full thickness slices should be 0.5-1.5 mm thick depending on joint location.
 - b. If harvesting whole joint, open joint in sterile hood, cutting ligaments and removing menisci and remove cartilage from tibial, femoral ad patellar surfaces.
 - c. If slices are large, use razor/scalpel blade to cut into pieces no larger than 3x3x1 mm.
- 2) OPTIONAL: Weigh tubes with tissue to determine amount of AC harvested.
- 3) Using forceps and spatula, transfer cartilage pieces into tube with stir bar and pronase solution, cap loosely **OR** remove complete PBS from 50 ml centrifuge tube and add pronase solution with stir bar, cap loosely.
- 4) Incubate 1 hr (37°C, 5% CO₂) on spinner plate (or shake every 15 minutes if unable to use spinner plate in incubator); cartilage will become sticky in this step and may stick to sides of bottle.

- 5) Remove pronase, taking care not to remove cartilage (if desired, you can centrifuge the solution you remove and resuspend any pellet in collagenase solution and return to bottle) and add collagenase solution.
- 6) Incubate overnight (maybe as short as 4-6 hours if you want to do it all in one day - all tissue should be digested) on spinner plate; make sure cap is loose!
- 7) Strain through 70 μm pore strainer into new 50 ml centrifuge tube.
- 8) Centrifuge (10 min to spin down cells).
- 9) Resuspend in \sim 30 ml complete media.
- 10) Count cells and assess viability (should have $10\text{-}20 \times 10^6$ cells/joint depending on size of canine with $>90\%$ viable if cap was left loose during digestion).
- 11) Spin (again) and resuspend at 2×10^6 cells/17 ml for monolayer culture OR 4×10^6 cells/ml for seeding into 9 mm diameter matrices OR 7.5×10^6 cells/ml for seeding into 4 mm diameter matrices.

APPENDIX E. CELL CULTURE AND CELL SEEDING

E.1 Monolayer Culture

For monolayer cultures, wait 3 days (or until cells are attached) before changing media

- 1) Plate chondrocytes at a density of 2×10^6 cells/T-75 flask
- 2) Add 15-17 ml cell culture media per flask
- 3) Change media every 2-3 days
- 4) Passage cells when confluent (~5-7 days)

E.2 Cell Seeding Of Matrices

Materials

Sterile forceps

Sterile pipettes

Sterile pipette tips

Pipetteman (10 μ l, 200 μ l)

Centrifuge tubes (50 and/or 15 ml)

2cc Cryotube (for 4 mm diameter disks)

Well plates coated with sterile 2% agarose (24-well plate for 4 mm diam disk; 12-well plate for 9 mm diam disks)

Complete DMEM/F12

1X antibiotics/antimycotics

25 μ g/ml ascorbic acid

10% FBS

Trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; Gibco #25300-062)

Matrix preparation

- 1) Cut to desire dimension (4mm or 9 mm diameter) with dermal punch.
- 2) EDAC cross-link
- 3) Rinse 2x in complete DMEM/F12
- 4) Aspirate media and transfer disks to tube
 - 2cc cryotube for 4mm diameter disks
 - 15 ml centrifuge tube for 9 mm diameter disks

Cell preparation

- 1) Aspirate and rinse media from cells with PBS (~5ml PBS/T-75)
- 2) Trypsinize (see Appendix H) cells from flasks (~3 ml trypsin/T-75; ~5 minutes)
- 3) Stop trypsinization with FBS containing media (~5ml/T-75)
- 4) Spin cell suspension; aspirate media; resuspend cells and take 10-100 μ l for cell count (see Appendix I)
- 5) Dilute cell count sample with trypan blue or erythrosin B vital dye – usually 1 part cell suspension:1 part vital dye (dilution factor=2)-1:4 (dilution factor=5) – and count cells (viable cells clear, dead cells blue with trypan blue, red with erythrosin B)

$$\# \text{ cells} = \frac{\# \text{ CellsCounted}}{\# \text{ SquaresCounted}} \times \text{DilutionFactor} \times \text{SuspensionVolume} \times 10^4$$

- 6) Spin cells and resuspend at desired concentration
7.5x10⁶ cells/ml for 4mm diameter disks
4x10⁶ cells/ml for 9mm diameter disks

Seed cells

- 1) Add cell suspension to matrices in tube and mix gently
- 2) 15 disks/ml for 4mm diameter disks
- 3) 2 disks/ml for 9mm diameter disks
- 4) Place on nutator in incubator for 1.5-2hrs
- 5) Transfer disks to agarose coated wells
- 6) Add media
0.5 ml for 4mm diameter disks in 24 well plates
- 7) Next day add additional media
To 1ml total volume for 4mm diameter disks
- 8) Change media every 2-3 days

APPENDIX F. COATING WELL PLATES WITH AGAROSE

- 1) Agarose coating prevents cells from attaching and growing on the bottom of well plates. This ensures that your explants or constructs will have all medium nutrients solely available to them. Each well of a 24-well plate is to be coated with 0.5 mL of liquid agarose or enough to evenly coat bottom of wells. Prepare 10-15 mL more agarose than you will need.
- 2) In a 100 mL glass bottle, place agarose and distilled water. Use 1 g Seaplaque agarose (#50100, BioWhittaker/FMC Bioproducts) per 25 mL water. (Or you can use 1 g of Biorad agarose per 50 mL water.)
- 3) Autoclave the bottle on the liquid setting in an autoclave bag with the cap untightened.
- 4) Remove the bottle from the autoclave while it is still hot.
- 5) Coat the wells with 2 mL of the liquid agarose each. This should be done rather quickly as the agarose will solidify as it cools.
- 6) Leave the well plates to solidify at room temperature for at least 2 hours or place in cold room to speed up the solidification, but warm in incubator 1-2 hours before transferring matrices. Often it is easiest to do the agarose coating the day before using the plates and leave in the incubator overnight to solidify.
- 7) Change agarose-coated well plates every two weeks because the agarose breaks down.

APPENDIX G. CHONDROCYTE SPECIFIC MEDIA

G.1 Complete Media

Materials

| | | |
|-----------------------|--------------------------|-------------|
| DMEM/F12 | Ascorbic acid solution** | Pipette Aid |
| Heat Inactivated FBS* | Sterile glass bottle | Flame |
| Antibiotics | Sterile pipettes | |

Methods

- 1) Warm all solutions for 30 minutes in a 37°C water bath.
- 2) Remove 65 ml of DMEM/F12 from the 500 ml bottle and place in a sterile bottle for later use.
- 3) Add 50 ml of heat inactivated FBS.
- 4) Add 5 ml of antibiotics.
- 5) Add 10 ml of ascorbic acid solution.

Complete medium is good for 2 weeks (due to inactivation of FBS with time). Do not prepare more medium than you will need for two weeks at a time.

***Heat Inactivation of FBS**

- 1) Turn on the 56°C water bath and let warm up.
- 2) Remove FBS from -20°C freezer (downstairs - general supply).
- 3) Place bottle in 56°C water bath. Once thawed, leave for an additional 30 minutes.

****Ascorbic Acid Solution**

- 1) Weigh out 0.0125 g of ascorbic acid for every 10 ml of ascorbic acid solution to be prepared.
- 2) For 50 ml, weigh 0.0625 g ascorbic acid and add 50 ml incomplete DMEM/F12.
- 3) Filter through a 0.45 µm sterile filter.
- 4) Aliquot into 15 ml tubes in 10 ml aliquots and store in -20°C freezer.

G.2 Changing Media

Materials

Complete Medium

Pipettes

Flame

Vacuum Pipettes

Vacuum setup

Methods

- 1) Remove the medium from the culture dishes or flasks using the glass vacuum pipettes (use long pipettes for flasks, short pipettes for well plates). Make sure to use a new pipette for each sample from different animals.
- 2) Replace the media according to the type of culture dish you are using.
 - a. 75 cm² flasks – 15 ml
 - b. 25 cm² flasks – 5 ml
 - c. 24 well plate – 1 ml per well
- 3) You can use the same pipette as long as you do not touch the flasks. If you think you might have contaminated it at all, use a new one. Be sure to switch pipettes for samples from different animals.
- 4) Quickly flame caps and lids before putting them back on to ensure they remain sterile.

APPENDIX H. Passaging Cells

Materials

Complete medium

Trypsin

PBS (Phosphate-buffered saline)

Sterile glass and plastic pipettes

Vacuum setup

Centrifuge tubes

Tissue culture flasks

Methods

- 1) Warm the medium, trypsin, and PBS in 37° C water bath.
- 2) Remove medium from flasks with vacuum pipette (change pipettes for different animals).
- 3) Rinse with PBS (enough to cover bottom of flask, ~ 10 ml for 75 cm² flask) to removal any residual medium. Trypsin will not detach the cells if it has come into contact with the medium.
- 4) Remove PBS and add trypsin/EDTA (2 ml for 25 cm² flask or 5 ml for 75 cm² flask).
- 5) Place in incubator for 3 - 5 minutes (unless otherwise instructed).
- 6) Remove from incubator and tap on the sides of the flask to loosen the cells. Check under microscope to ensure the cells are no longer attached. If they are, return them to the incubator and check each minute until they are unattached.
- 7) Once the cells are floating, return to the hood and add complete medium to inactivate the trypsin (3 mL for 25 cm² flask or 10 mL for 75 cm² flask).
- 8) Using a sterile plastic pipette, transfer the medium/trypsin/cell suspension to a centrifuge tube. At this point you can combine the contents of the flasks if they are from the same sample.
- 9) Balance the tubes and centrifuge at 1500 rpm for 10 minutes.
- 10) Once you have the cell pellet at the bottom of the tube, draw off the medium with the vacuum pipette (be sure not to suck up the cells!!!).
- 11) Resuspend and count the cells (Appendix I). While counting, centrifuge the cell suspension a second time to ensure all trypsin has been removed.

Decant medium from second centrifugation and resuspend at desired seeding density.

Transfer to culture flasks and add complete medium to bring the flasks up to final volume.

APPENDIX I. CELL COUNTING WITH A HEMOCYTOMETER

(modified by Vickers)

Materials

| | | |
|---------------------|----------------|-----------------------|
| complete medium | pipette aid | 70% isopropyl alcohol |
| trypan blue | micropipetters | cell counter |
| cell counting slide | pipette tips | |

Methods

- 1) Clean surface of hemocytometer and coverslip with 70% alcohol.
- 2) The coverslip should rest evenly over the silver counting area.
- 3) Beginning with a cell pellet, suspend the cells in a known amount of complete medium
- 4) Collect a 15 μ L sample from the cell suspension and put in microcentrifuge tube. Dilute with 15 μ L of trypan blue.
- 5) Mix well, and collect 15 μ L of suspension in a micropipette tip.
- 6) Load the cell suspension into the hemocytometer, allowing it to be drawn under the coverslip by capillary action. Load just enough cell suspension to reach the edges of the silvered surface. Do not overfill as this may change the volume and make the count inaccurate.
- 7) Place hemocytometer on microscope stage, remove yellow glass filter, and view with standard 10X objective.
- 8) Count cells in each of the four corner and central squares (clear cells are viable, blue stained cells are dead) (Figure I.1). Count cells that lie on the top and left lines but not those on the bottom or right lines of each square in order to avoid counting the same cells twice for adjacent squares. Repeat counts for other counting chamber. When a count of living cells is complete, count the number of dead cells stained blue in order to report viability.
- 9) Calculate total cell number from the following:

$$T = \frac{N_c}{N_s} \times D \times 10^4 \times V \quad \text{Equation I.1}$$

T = Total number of cells in suspension

N_c = Number of cells counted

N_s = Number of squares counted

D = Dilution factor, usually 2

V = Volume of media used to suspend cell pellet

- 10) The number 10^4 is the volume correction factor for the hemocytometer: each square is 1x1 mm and the depth is 0.1 mm.

- 11) Cell viability is calculated by:

$$\left(1 - \frac{\# \text{ dead}}{\# \text{ alive}}\right) * 100\% \quad \text{Equation I.2}$$

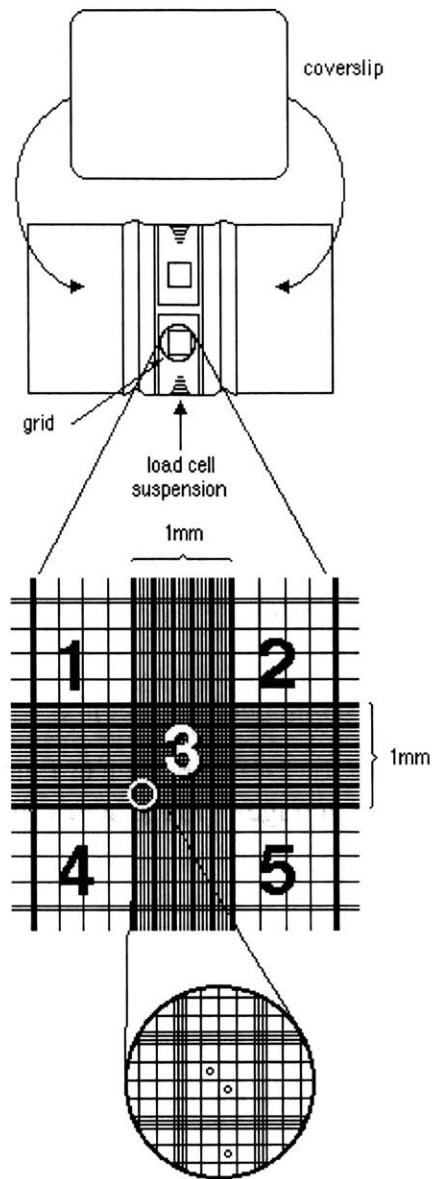


Figure I.1. Hemocytometer Counting Diagram

APPENDIX J. FREEZING AND THAWING CELLS

Materials

Complete medium

DMSO (Dimethyl sulfoxide): Autoclaved in light-proof bottle prior to use.

Sterile filter

Sterile pipettes

Sterile cryogenic tubes

Flame

Pipette Aid

15 ml centrifuge tubes

J.1 Freezing Cells

Methods

- 1) Determine amount of medium needed (1 mL per 2×10^6 cells) and add it to cells in 50 mL centrifuge tube.
- 2) Add 10% DMSO (i.e., if there is 15 mL of cell/medium suspension in 50 mL tube, add 1.5 mL of DMSO.)
- 3) Store in cryogenic tubes (3.3 ml per 5 ml tube).
- 4) Place in the -20°C freezer for 2-4 hours (longer time in this range is preferable), then transfer to -70°C for storage. (Make sure cell suspension is frozen before transferring to -70°C or cells will die)

J.2 Thawing Cells

Methods

- 1) Place cells directly into a 37°C water bath. Agitate gingerly while cells thaw for 40-60 seconds.
- 2) Slowly add warm medium to the tube until it is full. This insures that the cells thaw into the medium.
- 3) If not unthawed yet, put the caps back on the tube and gently agitate tube manually.
- 4) Transfer the cells to a 50 mL tube. Add more warm medium. Wash them clean of medium + DMSO for 10 minutes in the centrifuge.

APPENDIX K. LYOPHILIZATION OF MATRIX SAMPLES

Preparation of samples:

- 1) Rinse in matrices in PBS after culture and store in microcentrifuge tubes at -20°C until ready to lyophilize.
- 2) Before lyophilization, upon microcentrifuge tubes and place parafilm over opening. Poke holes in parafilm with a needle to allow moisture to escape, but still contain the matrix.

To start:

- 1) Pull knob on freeze dryer to empty any water in the chamber.
- 2) Turn on main switch.
- 3) Wait 10 min. until temperature is -50°C.
- 4) Turn on vacuum switch.
- 5) Close any open valves. Put everything on "VENT," and make sure no other valves are open.
- 6) Wait until pressure is in the green zone, about 5 min. Note that the display may be broken.
- 7) Attach jar with samples and turn the valve corresponding to the jar to "VAC."
- 8) Confirm that the temperature goes up.
- 9) Record operation on log sheet.

To end after overnight freeze-drying:

- 1) Turn the valve corresponding to the jar 90° to position in between "VAC" and "VENT."
- 2) Open a different valve to release the pressure.
- 3) Turn off the vacuum pump.
- 4) Turn off the main switch.
- 5) Gradually turn the valve corresponding to the jar to "VENT."
- 6) Remove the jar.
- 7) Measure the mass of the matrices the same day.
- 8) The matrices may be frozen for further use prior to proteinase K digestion, but they will absorb a small amount of water in that environment. Thus, mass measurements will not be accurate after freezing, but assays can still be performed.

APPENDIX L. PROTEINASE K DIGESTION OF SEEDED COLLAGEN MATRICES

Materials

Proteinase K (Roche # 745-723, for 100 mg bottle)

Tris-HCl buffer (1L): 0.05M Tris HCl (7.88 g/L)

1mM CaCl₂ (147 mg/L)

Dissolve above salts in 900 ml distilled water

Adjust pH to 8.0 with 1N NaOH

Bring to volume with distilled water

Methods

Stock Solution

- 1) Make up stock solution of 2-10 mg proteinase K/ml Tris-HCl buffer.
- 2) Resuspend proK powder in desired amount of Tris-HCl buffer
- 3) Transfer to 15 or 50 ml centrifuge tube and store at -20°C.

Working Solution

- 1) When ready to digest lyophilized samples, dilute stock solution to 0.1 mg/ml.
- 2) Add 1 ml of working solution to sample.
- 3) Incubate in water bath overnight (12-16 hours) at 60°C. Do not let water bath exceed 60°C because proK is inactivated at 65°C.
- 4) To aid in digestion, shake samples manually after 0.5-1 hour.
- 5) If samples aren't digested completely by 16 hrs, you can add a little more enzyme, but note the final volume of your samples.
- 6) After digestion, store samples at -20°C until ready to assay.

Note: Dilute GAG and DNA standards with Tris-HCl buffer not PBE (old protocols) because papain digestion was used.

APPENDIX M. GAG ASSAY USING DIMETHYLMETHYLENE BLUE (DMMB) DYE

DMMB dye binds to sulfated glycosaminoglycans and changes color from blue to purple based on concentration of GAG in solution (adapted from Farndale)

Equipment

LKB Biochrom Ultra Spec 4050 Spectrophotometer

Warm up for 30 minutes

Set wavelength to 525 nm

Turn Deuterium off

Blank the spec with cuvette (4.5 ml polystyrene cuvette) of air

Materials

48 mm acrylic cuvettes (#67.755 Sarstedt, Newton NC)

GAG standard - Stock concentration 1000 $\mu\text{g/ml}$

0.01 g shark chondroitin 6-sulfate

10 ml Tris-HCl

Sterile filter (0.45 μm)

DMMB dye solution

0.016 g 1,9 dimethylmethylene blue dye

5 ml 100% ethanol

Mix in 15 ml centrifuge tube to dissolve (2-3 hours)

2.37 g NaCl

3.04 g glycine

950 ml distilled water

Add dissolved DMB to solution

Rinse tube 2x w/100% EtOH and add wash to solution

8.7 ml 1N HCl

0.2 g sodium azide (toxic!!)

pH to 3.0 with 1N HCl

Bring to final 1L volume with water

Filter solution through large filter paper

OD reading at 525 nm (4.5 ml cuvette) should be in range 0.20-0.28 using cuvette of air as blank

Store in brown/amber bottle

Solution stable 3 months

Anything with DMMB dye is **ORGANIC WASTE!**

Methods

- 1) Serially dilute GAG standard.
- 2) Add 2 ml of DMMB dye and 20 μl of standard to cuvette (do not touch sides).
- 3) Set reference with 0.
- 4) Run Standards (0, 31.25, 62.5, 125, 250, 500, 1000 $\mu\text{g}/\text{ml}$) in triplicate and record absorbance.
- 5) Create standard curve (should be quadratic).
- 6) For digested samples (proK), add 100 μl of sample to 2 ml of DMMB dye and run in duplicate.
- 7) Record absorbance and determine GAG concentration from standard curve.

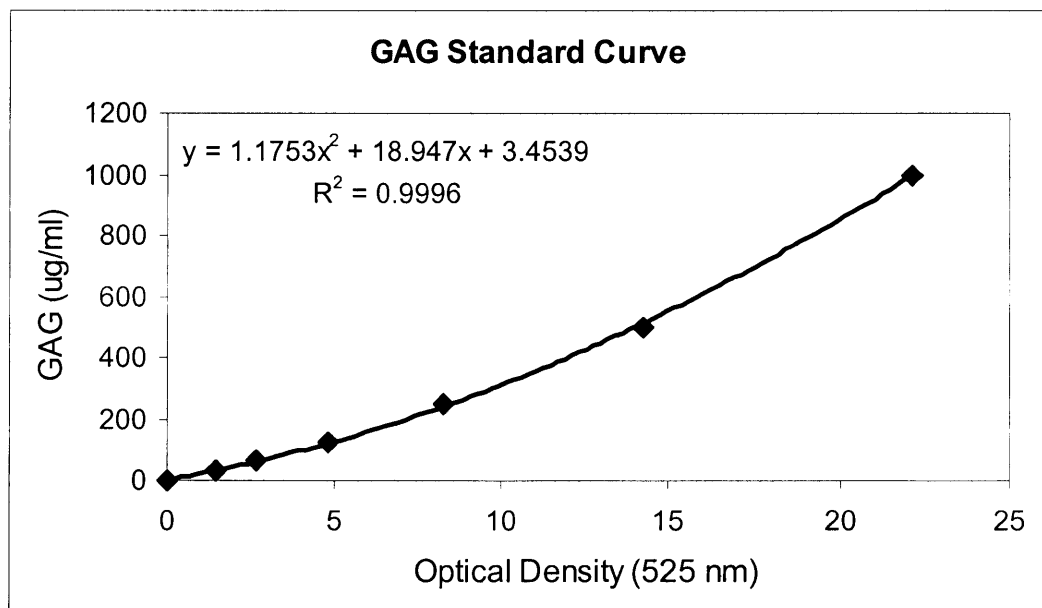


Figure M.1 Typical standard curve for GAG (shark chondroitin-6-sulfate) standards.

APPENDIX N. DNA ASSAY USING HOECHST 33258 FLUORESCENT DYE

(developed by Kim et al)

Bisbenzimidazole fluorescent dye Hoechst 33258 interacts with DNA dissociated from proteins of the nucleoprotein complex during standard papain digestion

Equipment

Hoefler Scientific Instruments TKO 100 Fluorometer

Excitation wavelength 365 nm

Emission wavelength 460 nm

Materials

48 mm acrylic cuvettes (#67.755 Sarstedt, Newton NC)

DNA standards - Stock concentration 1 $\mu\text{g}/\mu\text{l}$

1 mg calf thymus DNA

1 ml sterile Tris HCL

Hoechst dye stock solution - concentration 1 mg/ml

10 mg Hoechst 33258 dye

10 ml of dH₂O

DNA dye solution

450 ml distilled water

50 ml 10x TEN buffer

50 μl Hoechst 33258 dye (10000x) (light sensitive; carcinogenic)

Cover beaker in tin foil or use amber bottle to minimize exposure to light

TNE buffer (10x)

100 mM Tris

10 mM Na₂EDTA

1.0 M NaCl

pH 7.5

Anything with Hoechst dye is **ORGANIC WASTE!**

Methods

- 1) Serial dilutions in Tris HCl (for proteinase K-digested samples) to 5, 2.5, 1.25, 0.625, 0.3125, 0 $\mu\text{g}/\text{ml}$
- 2) Run standards (0, 0.3125, 0.625, 1.25, 2.5, 5, 10 $\mu\text{g}/\text{ml}$) in triplicate
 - a. 50 μl standard
 - b. 2 ml dye
 - c. Create standard curve (should be linear)

- 3) Run samples in duplicate
- a. 100 μ l sample
 - b. 2 ml dye
 - c. Determine DNA content from standard curve

Sample/dye complex should reach equilibrium in about 15 seconds and should be stable for at least 2 hours (less than 5% decrease in fluorescence)

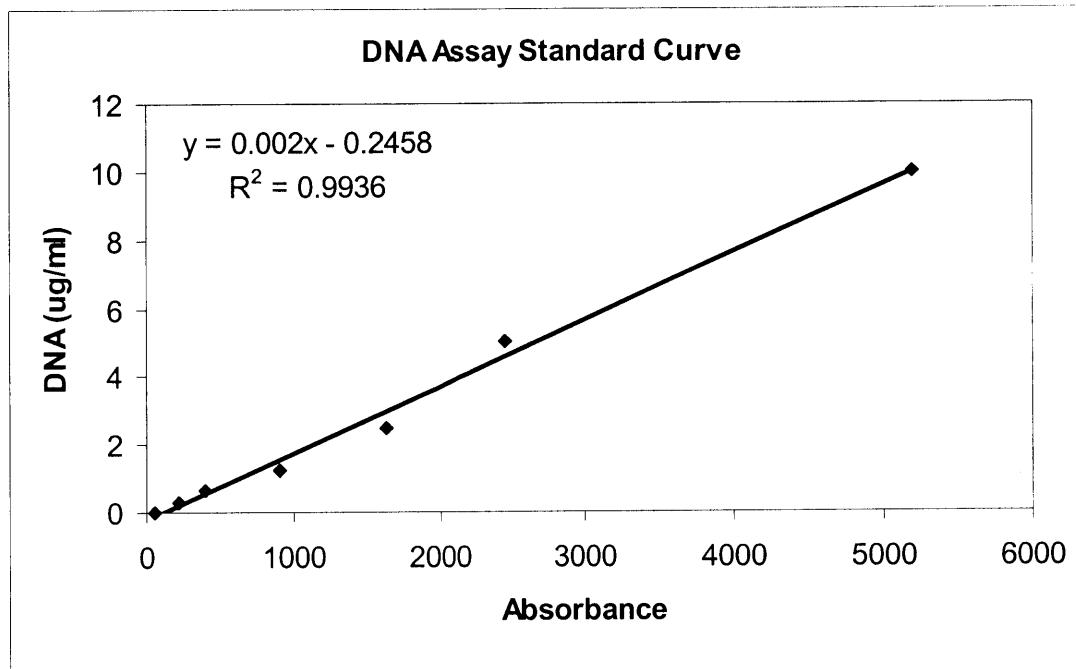


Figure N.1: Typical standard curve for DNA (calf thymus DNA) standards

APPENDIX 0. RADIOLABELING OF ³⁵S AND ³H PROTOCOL

Modified from Lee.

General

- 1) Label disks during final 24 hrs of culture with ³⁵S and ³H to measure GAG and collagen synthesis, respectively.
- 2) Wash unincorporated radiolabel.
- 3) Lyophilize tissue and measure dry weights.
- 4) Papain or Proteinase K digestion – use digest for GAG content, DNA content, and scintillation counts.

Materials Needed

- 1) Latex gloves (double glove)
- 2) Tape for labcoat sleeves
- 3) Aluminum foil to line hood
- 4) Complete medium (10% FBS)
- 5) Sterile 15 mL or 50 mL centrifuge tube
- 6) Pipetman – 20 µL and/or 200 µL
- 7) Sterile pipette tips – 200 µL capacity
- 8) Vacuum flask and sterile Pasteur pipettes
- 9) Radionuclides: tritiated proline (³H) (proline, L-[2,3,4,5-³H]; #NET,483, Perkin Elmer or #TRK534, Amersham Pharmacia Biotech, Inc.) and radioactive sulfate (³⁵S) (#NET041H, Perkin Elmer)
- 10) PBS
- 11) Na₂SO₄ (anhydrous sodium sulfate; #S421-1, Fisher Scientific Co.) and L-proline (P-8449, Sigma)
- 12) Clean spatula/forceps
- 13) 24 well culture plates
- 14) Sterile pipettes

Preparation of Radioactive Media

- 1) Calculate volume of isotope needed:
 - a. With 1 mL media/disc; $V=1n+2$ mL, where n is the number of discs.
 - b. ³⁵S has a half-life is 87.4 days:

$$\text{volume}^{35}\text{S} = \frac{R_{cS}V}{C_{vS} \times 2^{-x/87.4}}, \quad \text{Equation O.1}$$

where R_{cS} is the radioactivity concentration you desire for ³⁵S and C_{vS} is the concentration of radioactivity of your sulfate vial. The former (R_{cS}) is usually 5 µCi/mL for 4 mm-diameter CG discs. The latter is often 1000 µCi/mL when you order 1 mCi or 10,000 µCi/mL when you order 10 mCi, but you should check the bottle. x = # days past calibration date on vial.

- c. For ³H the half-life is 8-9 years.

$$\text{volume}^3\text{H} = \frac{R_{cH}V}{C_{vH}}, \quad \text{Equation O.2}$$

where R_{cH} is the radioactivity concentration you desire for ³H and C_{vH} is the concentration of radioactivity of your proline vial. The former (R_{cH}) is usually 5 µCi/mL for 4 mm-diameter CG discs. The latter is often 1000 µCi/mL when you order 1 mCi, but you should check the bottle.

- 2) Wipe hood with 70% EtOH.
- 3) Line with aluminum foil.
- 4) Double glove and tape lab coat sleeves.
- 5) Aliquot calculated volume of warm media into centrifuge tube.
- 6) Place radionuclide in hood on aluminum foil and loosen cap.
- 7) Use sterile technique to aliquot calculated volume of ^{35}S .
- 8) Save 1 mL of this single-labeled media for calibration.
- 9) Add calculated volume of ^3H .
- 10) Save 1 mL of this double-labeled media for calibration.
- 11) Remove pipette tips to original wrapper and dispose of in radioactive waste container.
- 12) Return radionuclides to container and note amount used; return to refrigerator.
- 13) Rinse Pipetman in cold water.
- 14) Check Pipetman and hood with Geiger counter.

Labeling of Discs

- 1) Place 1.5 mL of radiolabeled medium in 24 well plate (#08-772-1, Fisher Scientific Co.) wells. Generally, use the top row only so the bottom rows can be used for washings.
- 2) Use a forceps to transfer the discs to the radiolabeled wells. Drop them gently. Do not let the forceps touch the radiolabeled medium.
- 3) Return pipette to paper wrapper and dispose of in radioactive waste container.
- 4) After 24 hours, radiolabel is complete.

Washing of Discs

- 1) Complete PBS with 0.8 mM Na_2SO_4 and 1.0 mM proline.
 - a. For 500 mL PBS: 0.057 g Na_2SO_4 and 0.057 g proline.
 - b. Recommended to make fresh wash solution each time; can make frozen stock solutions of proline and sulfate and dilute in PBS.
- 2) Line hood with aluminum foil.
- 3) Aliquot 1 mL PBS/well in 24 well culture plate. Put this into all the wells below each radiolabeled well in the plate.
- 4) Place in cold room for 15 minutes.
- 5) Transfer disks to 3rd row and refrigerate for 15 minutes.
- 6) Repeat for a total of five washes.
- 7) Dispose of radiolabeled media in sink and note amount of radioactivity disposed.
Optional: Save the medium if you wish to count it.
- 8) Place each sample in a labeled vial.
- 9) Optional: Save PBS from last row (1 from each group) to make sure thoroughly washed (radioactive counts ~ background).
- 10) Lyophilize, measure dry weight, digest... (for DNA, GAG, scintillation counting).

APPENDIX P. SCINTILLATION COUNTING OF ^3H AND ^{35}S RADIOLABELED SAMPLES

Counting Protocol

- 1) Combine 100 μL of sample digest or calibrated media with 4 mL of scintillation fluid (ScintiVerse II; Fl-09-0797, Fisher Scientific Co.). Assay samples in duplicate.
- 2) Spray and wipe off the scintillation vials and holders with staticide (#2005, ACL Staticide). Then, queue them in the machine. The maximum number of samples at once is 230. The first vials go in the tray with 6 at the top. On this tray, turn the dial to 1 (means it counts the tray only once). The rest of the vials go in trays with no number. (Yellow dot on dial means do not count; white dot means count continuously.)
- 3) Count 1 minute/sample, with ^3H counts in channel A and ^{35}S counts in channel B. This is done by pressing the following sequence on the scintillation machine (Packard Tri-Carb 4640; Packard Instrument Co.):
 - End
 - Start
 - Enter 6
 - Enter
 - Make sure isotope line says 4
 - End
 - Forward and Enable simultaneously
4. After samples are counted, the vials with scintillation fluid and radioactive digest should be dumped into the barrel for liquid scintillation vials.

Calculations

Modified from Lee and Hastreiter

The amount of radioactivity, [^3H] or [^{35}S], is calculated based on the counts per minute (cpm) from channels A (C_1) and B (C_2) as follows:

$$\begin{pmatrix} [^3\text{H}] \\ [^{35}\text{S}] \end{pmatrix} = \begin{pmatrix} k_{11} & k_{12} \\ k_{21} & k_{22} \end{pmatrix} \begin{pmatrix} C_1 \\ C_2 \end{pmatrix} \quad \text{Equation P.1}$$

The coefficients k_{11} , k_{12} , k_{21} , and k_{22} are determined from the counts of the media samples taken when adding the isotopes to the media:

From the first media sample (^{35}S only, no ^3H) the matrix equation becomes: ([^{35}S] reflects concentration of ^{35}S added to media (i.e., 10 $\mu\text{Ci/mL}$); superscript “S” reflect that counts are from first media sample with ^{35}S only, subscripts denote the channel counted)

$$0 = k_{11}C_1^S + k_{12}C_2^S \quad \text{Equation P.2}$$

$$[^{35}\text{S}] = k_{21}C_1^S + k_{22}C_2^S$$

From the second media sample (^{35}S and ^3H) the appropriate equations are: ($[^{35}\text{S}]$ and $[^3\text{H}]$ reflect concentrations of ^{35}S and ^3H , in $\mu\text{Ci/mL}$, added to media, respectively; superscript "S,H" reflect that counts are from second media sample with both ^{35}S and ^3H , subscripts denote the channel counted)

$$[^3\text{H}] = k_{11}C_1^{S,H} + k_{12}C_2^{S,H}$$

$$[^{35}\text{S}] = k_{21}C_1^{S,H} + k_{22}C_2^{S,H}$$

$$\text{Equation P.3}$$

Solving these 4 equations for the 4 unknown k 's:

$$k_{11} = -k_{12} \frac{C_2^S}{C_1^S} = \frac{[^3\text{H}]}{C_1^{S,H} - \frac{C_2^{S,H}C_1^S}{C_2^S}}$$

$$k_{12} = \frac{[^3\text{H}]}{C_2^{S,H} - \frac{C_1^{S,H}C_2^S}{C_1^S}}$$

$$k_{21} = \frac{[^{35}\text{S}]\left(\frac{C_2^S}{C_2^{S,H}} - 1\right)}{\left(C_1^{S,H} \frac{C_2^S}{C_2^{S,H}}\right) - C_1^S}$$

$$\text{Equation P.4}$$

$$k_{22} = \frac{[^{35}\text{S}]\left(\frac{C_1^{S,H}}{C_1^S} - 1\right)}{\left(C_1^{S,H} \frac{C_2^S}{C_1^S}\right) - C_2^{S,H}}$$

Note that if you accidentally add the ^3H before the ^{35}S , these values change to:

$$k_{11} = \frac{[^3\text{H}]\left(\frac{C_2^H}{C_2^{S,H}} - 1\right)}{\left(C_1^{S,H} \frac{C_2^H}{C_2^{S,H}}\right) - C_1^S}$$

$$k_{12} = \frac{[^3\text{H}]\left(\frac{C_1^{S,H}}{C_1^H} - 1\right)}{\left(C_1^{S,H} \frac{C_2^H}{C_1^H}\right) - C_2^{S,H}}$$

$$\text{Equation P.5}$$

$$k_{21} = -k_{22} \frac{C_2^H}{C_1^H} = \frac{[^{35}\text{S}]}{C_1^{S,H} - \frac{C_2^{S,H}C_1^H}{C_2^H}}$$

$$k_{22} = \frac{[^{35}\text{S}]}{C_2^{S,H} - \frac{C_1^{S,H}C_2^H}{C_1^H}}$$

Use the sample values of C_1 and C_2 to determine the fraction of the total available

radiolabeled proline and sulfate that was converted into macromolecular form (fraction incorporated).

$$\alpha_{^3H} = \frac{C_1 k_{11} + C_2 k_{12}}{[^3H]}$$

$$\alpha_{^{35}S} = \frac{C_1 k_{21} + C_2 k_{22}}{[^{35}S]}$$

Equation P.6

There are 132 nmol/mL cold proline and 358.2 nmol/mL cold sulfate in complete DMEM/F12 media. (Numbers based on manufacture's values for DMEM/F12, the percentage of DMEM/F12 in complete medium--88%, and exclusion of any of these constituents from FBS--has not been measured.) The concentration of cold proline and sulfate far outnumbers the concentration of hot. These calculations assume that the same percent of radiolabeled proline/sulfate and unlabeled proline/sulfate was converted and that the amount of proline/sulfate added in radiolabeled form is insignificant compared to the concentrations of (unlabeled) proline/sulfate in the media. The amount of proline/sulfate incorporated (in nmol) into macromolecular form during the radiolabel period is then determined as follows:

$$\text{proline} = \alpha_{^3H}(132 \text{ nmol/mL})V$$

$$\text{sulfate} = \alpha_{^{35}S}(358.2 \text{ nmol/mL})V'$$

Equation P.7

where V is the volume of media fed to the cultures, in milliliters (i.e., 1.5 mL).

The values for the control matrices should be subtracted from the seeded matrices. Then, the incorporation data can be normalized to the time of radiolabel (i.e., 24 hours) and the amount of DNA in the construct to yield the rate of incorporation normalized to cell content (nmol/ μ g DNA/hr).

APPENDIX Q. MANUAL EMBEDDING OF MATRIX SPECIMENS

After rinsing, matrix only specimens should be fixed in formalin for 48 hours. Explant-matrix constructs should be fixed in formalin for at least a week.

- 1) Samples are gently placed in labeled Tissue Tek cassettes in between 2 blue sponges.
- 2) Dehydration. The cassettes are transferred between the following solutions for the designated time periods. All solutions are pre-made in buckets and kept in the flammables cabinet. The longer times in this protocol are for matrix-explant constructs; the shorter times are for matrices only.

| <u>Solution</u> | <u>Time</u> |
|----------------------------|---------------|
| distilled H ₂ O | 30 min-1 hour |
| distilled H ₂ O | 30 min-1 hour |
| 50% EtOH | 30 min-1 hour |
| 70% EtOH | 30 min-1 hour |
| 80% EtOH | 30 min-1 hour |
| 95% EtOH | 30 min-1 hour |
| 95% EtOH | 30 min-1 hour |
| 100% EtOH | 30 min-1 hour |
| 100% EtOH | 30 min-1 hour |
| 100% EtOH | 30 min-1 hour |

- 3) Paraffin embedding. Cassettes for paraffin (Paraplast Cat. #8889-501006, melting temp 56°C, Oxford Labware, St. Louis, MO) embedding proceed through additional solutions as follows:

| <u>Solution</u> | <u>Time</u> | <u>Temp</u> |
|-----------------|--------------|-------------|
| Histoclear | 1 min-2 hour | room temp |
| paraffin | 1 hour | 59°C |
| paraffin | 1 hour | 59°C |

- 4) Take care not to touch the cassettes when they have just been in Histoclear (Americlear histology clearing solvent, Baxter Healthcare Corp. #C4200-1, Deerfield, IL) because the solution will eat through gloves. The paraffin baths are located in the paraffin oven.

APPENDIX R. PARAFFIN EMBEDDING WITH TISSUE TEK MACHINE

1) Samples should be decalcified and rinsed well of fixative, and placed in plastic tissue cassettes (Tissue Tek unicassettes, #4170 or #4173, Miles, Inc., Elkhart, IN).

2) Dehydration and infiltration.
 Tissue specimens were dehydrated and infiltrated by machine (Tissue-Tek VIP 1000, model 4617, Miles Scientific, Mishwaka, IN). Dehydration program #4 was used, with solutions changed automatically as follows:

| Solution | Time | Temperature |
|-------------------|--------|-------------|
| 50% EtOH | 1 hour | room temp |
| 70% EtOH | 1 hour | room temp |
| 80% EtOH | 1 hour | room temp |
| 95% EtOH | 1 hour | room temp |
| 95% EtOH | 1 hour | room temp |
| 100% EtOH | 1 hour | room temp |
| 100% EtOH | 1 hour | room temp |
| 100% EtOH | 1 hour | room temp |
| clearing solution | 1 hour | room temp |
| clearing solution | 1 hour | room temp |
| paraffin | 1 hour | 59°C |
| paraffin | 30 min | 59°C |
| paraffin | 30 min | 59°C |

Clearing solution: Americlear histology clearing solvent, Baxter Healthcare Corp. #C4200-1, Deerfield, IL

Paraffin: Paraplast Cat. #8889-501006, melting temp 56°C, Oxford Labware, St. Louis, MO

3) Specimens are embedded with the aid of a Tissue-Tek II paraffin embedding center, model 4603 (Lab-Tek Products, Westmont, IL). The machine provides a molten source of paraffin and cooling plate. Paraffin is placed in stainless steel molds of varying size, the specimen placed in the mold, and the mold placed on a cooling plate. The tissue cassette is placed on the mold and additional paraffin is added to affix the plastic cassette to the mold.

APPENDIX S. HEMATOXYLIN AND EOSIN (H & E) STAINING

Sectioning and Storage

Histological sections are cut on a microtome (Reichert-Jung model 2050 Supercut, Leica Instruments, Nussloch, Germany). The thickness is 7 μm for paraffin sections and 5 μm for JB-4. The sections are placed on glass slides (Fisherbrand Superfrost Plus, Cat. #12-550-15, Fisher Scientific, Pittsburgh, PA). Slides with JB-4 sections are dried on a hot plate at low temperature for 5-30 min., and then stored at room temperature. Paraffin slides are placed in an oven at 50°C to melt off excess paraffin overnight, and then stored at room temperature.

Solutions

HEMATOXYLIN: Filter 200 ml of stock solution into staining dish. Sigma Harris Hematoxylin Solution, Catalog HHS-128, Modified: Hematoxylin, 7.5 g/L; sodium iodate, aluminum and ammonium sulfate, stabilizers and preservative.

ACID ALCOHOL: 200 ml of 70% ethanol (in dH₂O) + 0.5 ml HCl

AMMONIA WATER: 200 ml dH₂O + 5-10 drops ammonium hydroxide, generally 5 pH should be roughly around 10.0 - use pH paper.
Generally this should be mixed up fresh each day.

EOSIN: 100 ml stock solution + 100 ml dH₂O + 1.0 ml glacial acetic acid
Sigma Eosin Y Solution Aqueous, catalog HT110-2-128.

Paraffin Sections

1) DEPARAFFINIZE AND REHYDRATE

| | |
|--------------------|------------|
| Xylene: | 2 x 5 min. |
| 100% ethanol: | 10-20 dips |
| 100% ethanol: | 10-20 dips |
| 95% ethanol: | 10-20 dips |
| 80% ethanol: | 10-20 dips |
| 70% ethanol: | 10-20 dips |
| dH ₂ O: | 10-20 dips |

2) Harris hematoxylin, 10 min.

3) Rinse in tap water, approx. 1 min. running or swishing until almost clear

4) Acid alcohol. Dip quickly 5-10 times. 20-30 sec total.

5) Rinse in tap water. Until foaming stops, maybe 30 sec.

- 6) Ammonia water. Quick dips (5 or so) until blue.
- 7) Rinse in tap water. About 1 min.
- 8) Eosin, 45 sec.
- 9) Rinse in tap water, 2 min.
- 10) DEHYDRATE
 - 70% ethanol: 10-20 dips
 - 80% ethanol: 10-20 dips
 - 95% ethanol: 10-20 dips
 - 100% ethanol: 10-20 dips
 - 100% ethanol: 10-20 dips
 - Xylene: 2 x 5 min.
- 11) Air dry, coverslip with Cytoseal (Cytoseal 60; #18006, Electron Microscopy Sciences).

APPENDIX T. SAFRANIN-O STAINING

- 1) Deparaffinize and rehydrate:

| | |
|-------------------|---------------|
| xylene | 2 x 5 minutes |
| 100% EtOH | 10-20 dips |
| 95% EtOH | 10-20 dips |
| 90% EtOH | 10-20 dips |
| 80% EtOH | 10-20 dips |
| 70% EtOH | 10-20 dips |
| dH ₂ O | 10-20 dips |
- 2) Stain 10 minutes in Safranin-O (0.2 g Safranin-O, 1 ml acetic acid, 100 ml distilled water)
- 3) Rinse with tap water
- 4) Counterstain 10-15 seconds with Fast-Green (stock solution, dilute 1:5 for working solution: 0.2 g Fast Green, 1 ml acetic acid, 100 ml distilled water)
- 5) Dehydrate (70%, 80%, 90%, 95%, 100%, 100% EtOH, xylene)
- 6) Air dry sections
- 7) Coverslip with Permount

Glycosaminoglycans stain red/pink and collagen stains green.

APPENDIX U. GETTING DIGITAL IMAGES FROM MICROSCOPE

- 1) Turn on camera via green button on R.
- 2) Get the SmartCard from the left top drawer. Put the card in the upper slot on the camera's left. The card takes images numbered from 110 down.
- 3) Switch on the screen.
- 4) Put the right and left knobs on the microscope to TV settings.
- 5) Hit the preview button the camera controller. This shows what the actual image recorded will be. Toggle the preview button for fine focusing.
- 6) Lighten/darken the image with the front turning dial or the back left knob on the microscope.
- 7) Press expose on the camera controller to take the picture.
- 8) If something seems odd, check the menu settings of the camera. They should be:
auto
SHQ
1st drive
100 ISO
normal
off
setup 5s
SQ 1280 x1024 JPEG
name reset
reset yes

The setting visible on the camera controller screen should be SM (can change with the memory button), record auto.

- 9) Getting the image off of the Smartcard:
 - a. Put the card in the flashcard reader.
 - b. Open Flash Reader on the computer.
 - c. Open DCIM/100olymp.
 - d. Images will be number as 1-110. These actually correspond to 110-1 when you were taking the pictures.
 - e. Open H drive and put your images there.
 - f. Put the Smartcard image files in the recycle bin.
- 10) Note that there are X2.5 and X3.3 objectives for the camera. Note which is being used.
- 11) Take images of the ruler slide to get a scale bar for your images.

APPENDIX V. Western Blot – Type I or Type II Collagen – Chemiluminescence Method

(modified from H.-H. Hung, MIT Continuum Electromechanics Lab and Scott Vickers, ORL - BWH)

Materials

| | | | |
|--------------------------------------|-------------------|----------------------------|-----------------|
| 2 mercaptoethanol | Sigma M-7154 | Type II Antibody (primary) | Iowa C11C1 |
| 20X Lumiglo 7003 | Cell Signal Tech. | Type I Antibody (primary) | Sigma C-2456 |
| Acrylamide | Sigma | PVDF Membranes | BioRad 162-0176 |
| Ammonium Persulfate | Fisher A-682-500 | Secondary Antibody | Sigma A-2304 |
| BIS | Sigma | TEMED | Sigma T-9281 |
| Bromophenol Blue | | Tris Buffered Saline (TBS) | Sigma T-6664 |
| Coomassie Blue | Sigma B-8522 | Trizma Base | Sigma T-1503 |
| Dry Milk | BioRad | Tween 20 | |
| Filter Paper (for blots) | BioRad 162-0118 | X-ray Film | Sigma F-5513 |
| Glycerol | Sigma G-8773 | Glycine | Sigma G-8898 |
| Urea | | 5 mM DDT | |
| Laurel Sulfate- Sodium Salt (SDS) | Sigma L-4509 | | |

Prepared Solution

30% Acrylamide-bisacrylamide

| | |
|-------------------|--------|
| Acrylamide | 29.2 g |
| BIS | 0.8 g |
| dH ₂ O | 100 ml |

Note: Filter through #1 Whatman filter paper, store at 4°C. Good for 1 month, store in brown bottle or foil covered bottle

10% Ammonium Persulfate

| | |
|---------------------|-------|
| Ammonium Persulfate | 0.1 g |
| dH ₂ O | 1 ml |

Note: Store at 4°C, good for 1 week.

Stacking Buffer (pH 6.8)

| | |
|-------------------|--------|
| Tris base (0.5M) | 7.9 g |
| dH ₂ O | 100 ml |

Note: Filter through #1 Whatman filter paper, store at 4°C. Good for 1 month.

Resolving Buffer(pH 8.8)

| | |
|-------------------|--------|
| Tris base (3.0M) | 46.8 g |
| dH ₂ O | 100 ml |

Note: Filter through #1 Whatman filter paper, store at 4°C. Good for 1 month.

Sample Buffer(2x stock solution)

| | |
|--------------------------|--------|
| Tris base (0.5M, pH 6.8) | 1.0 ml |
| Glycerol | 0.8 ml |
| 10% SDS | 1.6 ml |
| 2ME | 0.4 ml |
| Dye | 0.2 ml |

Notes: 1. Store at -20°C
2. 2ME = 2 mercaptoethanol, in hood in biochem lab
3. Dye = 1% bromophenol blue

Running Buffer (10x stock solution)

| | | |
|-------------------|---------|---|
| Tris base | 30.3 g | Note: pH = 8.3, Store at room temperature |
| Glycine | 144.0 g | |
| SDS | 10.0 g | |
| dH ₂ O | 1.0 L | |

Transfer Buffer (5x stock solution)***

| | | |
|-------------------|---------|--|
| Tris base | 15.15 g | Notes: 1. Store at room temperature |
| Glycine | 72.25 g | 2. ***For dilution to 1x, add 20% |
| dH ₂ O | 800 ml | (160 ml 5X buffer, 480 ml dH ₂ O, 160 ml methanol). |

TBS-T

| | |
|----------|--------|
| Tween 20 | 1.0 ml |
| TBS | 1.0 L |

Defixation Buffer

| | |
|-------------------|-----------------------------|
| 6 mM Urea | 0.036 g |
| 192 mM glycine | 1.41 g |
| 25 mM Tris | 0.303 g |
| 0.2% SDS | 0.20 g |
| 5 mM DTT | 0.077 g (stored in freezer) |
| dH ₂ O | 100 ml |

V.1 SDS PAGE

Methods

1. Assemble the gel-casting apparatus. Fill ice tray for blot transfer and place in freezer.
2. Prepare the resolving gel. Load 7 ml of gel solution in each cassette. Overlay with a small amount of dH₂O.

Amounts will vary depending on the %T of the gel you desire. For the Type II Western, use 7%. Amounts are enough for 2 gels – dispose of extra in trash, not the sink

| | | |
|----------------|-------------------|---|
| Resolving gel: | dH ₂ O | 5.0 ml |
| (7%) | 30% Acryl-bis | 2.33 ml |
| | Resolving buffer | 2.5 ml |
| | 10% SDS | 100 µl |
| | 10% AP | 100 µl |
| | TEMED | 10 µl <i>Add TEMED under fume hood.</i> |

3. Begin boiling water for samples.

4. Prepare samples, control, and marker. For samples (collagen matrices) add 150 μ l of 1x sample buffer. For each standard (type I and type II), combine 2 μ l with 20 μ l of 1x sample buffer. For the molecular weight standard add 4 μ l with 20 μ l of 1x sample buffer. *Add sample buffer under fume hood*

NOTE: For type I and type II standard, if using new bottle, add 0.5M acetic acid to dry powder until desired concentration is reached. For example, you want 10 μ g of standard to be loaded so therefore if you have a 10 mg bottle add 2 ml acetic acid to have concentration of 10 μ g in 2 μ l. (Fisher FA4P0-212 acetic acid is 17.4 M)

5. When the resolving gel is set (approx. 20-30 min.), remove layer of dH₂O using filter paper.
6. Prepare the stacking gel. Load stacking gel solution and insert combs.

| | |
|---------------------------------|---|
| Stacking Gel: dH ₂ O | 5.6 ml |
| 30% Acryl-bis | 1.25 ml |
| Stacking buffer | 2.5 ml |
| 10% SDS | 100 μ l |
| 10% AP | 100 μ l |
| TEMED | 10 μ l <i>Add TEMED under fume hood</i> |

7. Heat the samples in boiling water for 10 minutes, then centrifuge in cold room for 10 minutes at the highest speed (14,000rpm).
8. Once the gels are solid, remove the combs and move the cassettes to the gel-running chamber.
9. Fill the inside of the chamber with 1x running buffer. Fill the outside of the chamber with the buffer to at least the first set of knobs.
10. Use the special loading pipette tips to load the samples in the gels.

Before loading samples, fill each well with running buffer to remove any bubbles.

11. Run the gel for approx. 2 hr. at 90V.

It is finished when the sample buffer has reached the bottom of the gel, so check periodically.

12. Remove the gel by using one of the spacers to carefully pry the glass plates apart. Cut away the stacking portion of the gel. Mark the gel in a way that such that the orientation will be preserved.

V.2 BLOT TRANSFER

Methods

1. Cut nitrocellulose membranes and filter paper to same dimensions as gel.
2. Briefly wet membranes in transfer buffer.
Be sure not to touch membranes with bare hands as oil from the hands can block transfer.
3. Incubate gel for 30-45 min in a defixation buffer.
4. Soak the gel, filter paper, and sponges in 1x Transfer buffer (** add methanol, see 1st page**) for 10 minutes to equilibrate the gel.

Be sure to keep track of which gel is #1 and #2. Gel equilibration is required to prevent a change in the size of the gel during transfer.

5. Assemble the transfer sandwich according to the following diagram. Keep the cassette submerged in transfer buffer to avoid air bubbles and prevent the membrane from drying. Gently rub out any bubbles between the gel and the membrane using a stir rod.

| |
|-------------------|
| Clear/White Plate |
| Sponge |
| Filter Paper |
| Membrane |
| Gel |
| Filter Paper |
| Sponge |
| Black Plate |

6. Load the cassette into the transfer chamber. Black faces black.
7. Add the remaining 1x Transfer buffer for a total of 1 L.
8. Run for 1 hour at 100 V.
9. Remove membranes and wash in TBS buffer for 5 minutes.
10. Stain gels with Coomassie Blue to verify transfer of proteins to the membrane.

Membranes can be dried and stored in resealable plastic bags at 4°C for 1 year or longer at this point. Prior to further processing, dried PVDF membranes must be placed into a small amount of 100% methanol to wet the membrane, then in distilled water to remove the methanol. (1. Current Protocols in Cell Biology)

11. If proceeding to immunoblotting and detection the following day, place membranes in blocker (5% dry milk) overnight in the cold room.

To prepare blocker: 5g dry milk to 100 ml TBS. Mix well.

V.3 IMMUNOPROBING AND VISUALIZATION OF BLOTTED PROTEINS

Methods - Probing

1. After blocking overnight in 5% dry milk.
 2. Incubate with primary antibody for 2 hours at room temperature on rocker.
For collagen type II antibody (Iowa C11C1, 1:5 – 1:100 dilution in 1% BSA/TTBS) – recommend 1:5 dilution
For example: 1:100 – add 0.2 ml primary to 20 ml 1% BSA/TTBS
1:50 – add 0.1 ml primary to 20 ml 1% BSA/TTBS
For collagen type I antibody (Sigma #C-2456, 1:2000 dilution in 1% BSA/TTBS)
- Be sure to use enough solution to keep membrane covered

3. Rinse in 1x TBS-T for 10 minutes, 3 times.
4. Incubate with secondary antibody for 1 hour at room temperature on rocker.
1:2,000 dilution.
5. Rinse in 1x TBS-T for 10 minutes, 3 times.

Methods - Detection

1. Prepare LumiGlo solutions A and B. You need a 1 ml of each solution for each membrane. Do not mix A and B together until you are ready to use them. Once washing is complete, thoroughly mix A and B together and put 2 ml on each membrane. For stronger bands when exposes you can use 200 μ l of A and B.
 - i) Mix 100 μ l A to 2 ml dH₂O.
 - ii) Mix 100 μ l B to 2 ml dH₂O.
2. Incubate for 2 minutes at room temperature.
3. Wrap membranes in saran wrap, carefully smooth out any air bubbles
4. Expose to film in dark room (Thorn 1224C) and develop. Adjust exposure time as needed.