7

Tissue Engineering of Articular Cartilage

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1. INTRODUCTION

Articular cartilage has a limited intrinsic capacity for repair [Mankin, 1982; Buckwalter and Mankin, 1997]. In experimental studies on adult animals, defects, extending through the full thickness of cartilage into the subchondral bone, heal with the formation of fibrocartilaginous repair tissue [Hjertquist and Lemperg, 1971; Shapiro et al., 1993; Wei and Messner, 1999]. Such repair tissue differs from normal cartilage in that it contains relatively low amounts of type II collagen and aggrecan, functionally important markers of the chondrocytic phenotype [Benya and Shaffer, 1982], and a relatively high amount of type I collagen, a fibrillar molecule not present in measurable amounts in normal adult articular cartilage [Eyre et al., 1992]. Associated with the abnormal composition of such repair tissue is a deficiency in biomechanical function [Athanasiou et al., 1995; Wei et al., 1998; Dounchis et al., 2000]. Because of the ineffectiveness of intrinsic repair, methods for stimulating cartilage repair are under active investigation [Sah, et al., 2005].

An underlying strategy of a variety of experimental and clinical methods to enhance repair of an articular cartilage defect is the introduction or recruitment of an appropriate cell population that can undergo chondrogenesis. Recruitment of endogenous chondroprogenitor cells from bone marrow has been achieved by penetration through the base of cartilage defects into the subchondral vasculature by subchondral drilling [Muller and Kohn, 1999], microfracture [Steadman et al., 2001], abrasion [Johnson, 2001], and spongialization [Ficat et al., 1979]. Alternatively, after multiplication of cells ex vivo, autologous chondrocytes have been introduced directly into a cartilage defect and secured with a periosteal flap [Brittberg et al., 1994]. The process of introducing and securing transplanted cells within the defect site may be simplified surgically, and accelerated temporally, by the use of preformed, cell-laden tissues. Although used infrequently, fresh osteochondral allografts, with fully formed and mature articular cartilage, have a long history of clinical success [Bugbee and Convery, 1999; Aubin et al., 2001], and, as a consequence, autologous osteochondral grafts have been introduced [Hangody et al., 2001]. Other chondrogenic tissues, such as periosteum [O'Driscoll and Fitzsimmons, 2001] and perichondrium [Bouwmeester et al., 1999], have also been implanted with the goal of promoting the differentiation of chondrogenic cells within the tissue into chondrocytes capable of producing a cartilage matrix. Likewise, cartilaginous tissues, engineered in vitro, may be suitable for repairing articular defects.

2. CULTURE CONDITIONS FOR CHONDROCYTES

Normally, chondrocytes within cartilage express a phenotype that is characterized by cellular secretion of components, including type II collagen and aggrecan, that are typically present in cartilage and comprise the cartilaginous extracellular matrix [Benya and Shaffer, 1982]. However, when cultured in monolayer under conditions that are considered standard for a variety of cell types, chondrocytes dedifferentiate to a fibroblastic phenotype. The dedifferentiated phenotype is characterized by the abnormal secretion of type I collagen and the reduced secretion of type II collagen and aggrecan. The propensity to dedifferentiate is especially marked when cultures are initiated at a low density and stimulated to proliferate. Compared to the culture of chondrocytes in monolayer, culture of chondrocytes in three-dimensional gel materials promotes the retention and restoration of the chondrocytic phenotype.

When cultured in alginate, chondrocytes can express stably, or be induced to redifferentiate from a dedifferentiated state, the chondrocytic phenotype. Alginate culture has such an effect on chondrocytes not only from animals but even from adult humans, with a stable phenotype having been maintained for as long as 8 months [Häuselmann et al., 1992]. Such cultures accumulate two distinct cartilage matrix components, termed the cell-associated matrix (CM) and the further-removed matrix (FRM). The presence of alginate gel promotes the retention of newly synthesized proteoglycan and collagen around cells, for example, compared to monolayer culture [Mok et al., 1994]. The CM forms rapidly around the cells, and, with time, the FRM forms further away from the cells. The cells with their CM can be recovered from alginate beads cultured for 1–2 weeks by dissolving the alginate polymer with agents that chelate divalent cations [Mok et al., 1994]. A number of in vitro studies have characterized the properties of matrix components and the regulation of their metabolism in this culture system [Häuselmann et al., 1992, 1994; Mok et al., 1994; Petit et al., 1996].

3. PREPARATION OF MEDIA AND REAGENTS

3.1. Media

3.1.1. Isolation Medium

Sterile DMEM-F-12, 50 μg/ml gentamicin, 360 μg/ml L-glutamine.

3.1.2. Culture Medium

Sterile DMEM-F-12, 50 μg/ml gentamicin, 360 μg/ml (2.5 mM) L-glutamine, 25 μg/ml ascorbic acid. Usually with 10% FBS, but sometimes 20% or serum-free culture medium as indicated in the text.

3.2. Saline Wash

Sterile NaCl, 0.9% (0.15 M), with 100 μg/ml gentamicin

3.3. Enzymes

3.3.1. Pronase Digestion Solution

(i) Prewarm the isolation medium to 37 °C in a sterile 50-ml centrifuge tube.

(ii) Mix the total amount of Pronase enzyme needed (0.10 g or 0.20 g) with 50 ml prewarmed isolation medium.

(iii) Dissolve for at least 1 hour.

(iv) Filter sterilize the Pronase enzyme digestion solution, using a 0.22-μm sterile Millipore Steriflip-GP 50-ml filter unit or Steritop-GV Filter Unit (150 ml).

Use 0.2% Pronase for bovine cartilage and 0.4% Pronase for adult human cartilage.

Total volume of Pronase enzyme digestion solution needed: ~50 ml/8 g of cartilage to be digested.

3.3.2. Collagenase-P Digestion Solution

Prepare a 2× collagenase-P enzyme digestion solution as follows:

(i) Prewarm 25 ml culture medium to 37 °C in a sterile 50-ml centrifuge tube.

(ii) Mix the total amount of collagenase-P enzyme needed (12.5 mg per 25 ml) with serum-free culture medium.

(iii) Dissolve for at least 1 h.

(iv) Filter sterilize the 2× collagenase-P enzyme digestion solution, using a 0.22-μm sterile Millipore Steriflip-GP 50-ml filter unit.

Total volume of the final 1× Collagenase-P enzyme digestion solution needed: ~50 ml/8 g of cartilage to be digested (the same as for the Pronase digestion).

3.3.3. Papain Enzyme Digestion Solution

Papain, 20 μg/ml in 0.1 M sodium acetate (NaAc), 0.05 M EDTA, pH 5.53. Prepare as follows:

(i) Prepare papain buffer solution—0.1 M NaAc, 0.05 M EDTA, pH 5.53

NaAc (MW 82.03)	4.102 g
EDTA (MW 372.2)	9.360 g
UPW	500 ml

Add UPW to less than 500 ml. Adjust the pH to 5.53 and then add UPW to a final volume of 500 ml. Store at 4 °C.

(ii) Prepare papain enzyme stock—23 mg protein/ml in UPW.

(iii) Just before use, activate the papain buffer solution by adding 5 mM L-cysteine hydrochloride hydrate (21.95 mg L-cysteine/25 ml of papain buffer).

(iv) Withdraw 5 ml for use with the controls.

(v) Add 17.4 μl 23 mg protein/ml papain enzyme stock to 20 ml activated (with L-cysteine) papain buffer to make a 20 μg/ml papain enzyme digestion solution.

Note: The concentration of protein per milliliter for each lot of papain enzyme changes. Therefore, the amount of papain enzyme to add must be calculated for each lot (bottle) of enzyme.

3.3.4. Pepsin Stock Solution

Pepsin, 1 mg/ml in 0.05 M acetic acid

3.3.5. Pancreatic Elastase

1 mg/ml stock solution of pancreatic elastase in high-salt Tris buffer (See Section 3.6.8.)

3.4. Alginate Encapsulation

3.4.1. Alginate Solution

Sodium alginate, 1.2% in 0.9% (0.15 M) NaCl; ***prepare fresh***

0.9 g	Alginate
75 ml	0.9% (0.15 M) NaCl, sterile solution

(i) Add 75 ml of 0.9% NaCl, sterile solution, to a sterile sample cup along with a sterile stir bar.

(ii) Add 0.9 g alginate.

(iii) Stir for at least 3 h to dissolve.

(iv) Once dissolved, filter the solution through a sterile 0.22-μm Millipore Steriflip-GP 50-ml filter unit. Do not autoclave the alginate solution.

3.4.2. Alginate Dissolving Buffer

Without EDTA: sodium citrate, 0.055 M, in 0.15 M NaCl, pH 6.8.

Na citrate (MW = 294.1)	16.2 g
NaCl (MW = 58.44)	8.8 g
UPW	1 L

With EDTA: sodium citrate, 0.055 M, in 0.03 M EDTA, 0.15 M NaCl, pH 6.8

(i) Add UPW to less than 1 L.

(ii) Adjust the pH to 6.80 and then add UPW to a final volume of 1 L.

(iii) Filter through a sterile 1-L bottle filter system.

(iv) Store at 4 °C.

3.4.3. Calcium Chloride Solution

$CaCl_2$, 102 mM

$CaCl_2$ (MW = 147.0)	15 g
UPW	1 L

(i) Dissolve and then filter through a sterile 1-L bottle filter system.

(ii) Store at 4 °C

3.5. Proteoglycan Assays

3.5.1. Proteoglycan Standard Stock

Proteoglycan, 2 mg/ml in 0.05 M NaAc and 0.05% Tween 20 in UPW, pH 6.8
Aliquot and freeze.

3.5.2. Proteoglycan Dilution Buffer

NaAc, 0.05 M, with 0.05% Tween 20, at pH 6.8

3.5.3. Dimethylmethylene Blue (DMMB) Dye Reagent

DMMB, 16 μg/ml, in 0.03 M sodium formate, 0.2% V/V formic acid, pH 6.8.
To prolong the activity of the DMMB dye, it is desirable to first solubilize the reagent in organic solvents before diluting it into a mostly aqueous environment.

(i) Dissolve 16 mg DMMB dye in 5.0 ml absolute ethanol. Solubilize for 15 min at room temperature (RT).

(ii) Add 2.0 ml formic acid and 2.0 g sodium formate to approximately 20 ml UPW. Dissolve and add to the ethanol-DMMB solution.

(iii) Stir for 20 min at RT.

(iv) Add UPW to less than 1 L.

(v) Adjust the pH to 6.80.

(vi) Add UPW to a final volume of 1 L.

Note: By adjusting the pH before making the final volume 1 liter, the dye solution should be more reproducible.
Do not filter or refrigerate the DMMB reagent!
DMMB activity is transient, so shelf life is only 1 month.

3.5.4. Guanidine Hydrochloride (GuHCl)

Prepare a 2.88 M GuHCl stock solution in 0.05 M NaAc pH 6.8 as follows:

(i) Prepare a 0.078 M NaAc pH 7.0 solution

NaAc (MW = 82.03), 0.078 M 6.40 g/L

(ii) For 1 L, mix the 0.078 M NaAc pH 7.0 solution with an 8 M GuHCl solution as described below:

GuHCl, 8 M	360 ml
NaAc, 0.078 M, pH 7.0	640 ml

(iii) The pH should be measured after mixing to validate that it is 6.8.

3.6. Collagen Assays

3.6.1. Hydroxyproline Drying Solution

TEA-methanol: methanol:dH_2O:triethylamine (TEA) (2:2:1)

Methanol	500 μl
Deionized water	500 μl
TEA	250 μl

Store at 4 °C
This will yield enough drying solution to dry 25 samples twice.

3.6.2. Hydroxyproline Derivatizing Solution

Methanol:dH_2O:TEA: phenylisothiocyanate (PITC)—7: 1:1:1; ***prepare fresh***.

(i) For 25 samples prepare 500 μl derivatizing solution. Always make in excess, so for 25 samples, prepare 600 μl total.

Methanol	420 μl
TEA	60 μl
Deionized water	60 μl

(ii) Finally, add 60 μl PITC to the above solution.

△ ***Safety note.*** **PITC IS VERY HARMFUL. WEAR GLOVES AND DO THIS STEP IN A FUME-HOOD**. Any excess derivatizing solution that is not used with samples should not be used again and should be stored at −20 °C until disposal. This should be treated as hazardous waste for disposal purposes.

Note: Unopened vials of PITC are stored at RT. Once opened, any unused PITC should first be "bubbled" under nitrogen, to remove all air, and then the vial should

be stored at −20 °C. PITC treated in this manner is good for use for no longer than 3 weeks. For disposal, expired, unused PITC should be treated as hazardous waste.

3.6.3. Hydroxyproline HPLC Standard

Hydroxy-L-proline, 0.2 mg/ml:

Hydroxy-L-proline	10 mg
HPLC-grade water	50 ml

(i) Prepare 100-µl aliquots and lyophilize.

Standard concentration: 20 mg/tube; 20 ng/µl.

Lyophilized standard is derivatized simultaneously with samples and then reconstituted in 1 ml of reconstitution buffer.

3.6.4. Collagen Reconstitution Buffer

Na_2HPO_4, 4.75 mM in 5% V/V acetonitrile, pH 7.4.

(i) Prepare buffer: sodium phosphate, dibasic, anhydrous (Na_2HPO_4), 5 mM

Na_2HPO_4 (MW = 142.0)	710 mg
HPLC-grade water	1 L

(ii) Titrate to pH 7.4 with 10% V/V phosphoric acid (H_3PO_4)

(iii) Add acetonitrile:

Acetonitrile	50 ml
Na_2HPO_4 buffer pH 7.4, 5 mM	950 ml

(iv) Store at 4 °C

3.6.5. Hydroxyproline HPLC Eluent A

Acetonitrile:dH_2O:140 mM sodium acetate trihydrate buffer (6:4:90), pH 6.4 with 0.5 ml/l TEA.

(i) Prepare 140 mM sodium acetate trihydrate-TEA solution

Sodium acetate trihydrate (MW = 136.08)	38 g
HPLC-grade water	1800 ml

(ii) Once dissolved, add 1 ml TEA

(iii) Titrate to pH 6.4 with glacial acetic acid

(iv) Bring resulting solution to 2 L in a 2-L volumetric flask with HPLC-grade water

(v) In a separate 2-L glass bottle add:

Acetonitrile (CH_3CN)	120 ml
Sodium acetate trihydrate, 140 mM-TEA solution	1880 ml

(vi) Store at 4 °C

(vii) Before use, de-gas in a sonicator.

3.6.6. Hydroxyproline HPLC Eluent B

60% Acetonitrile:

Acetonitrile	600 ml
HPLC-grade water	400 ml

(i) Prepare in a glass bottle.

(ii) Store at 4 °C

(iii) Before use, de-gas in a sonicator.

3.6.7. Collagen Extraction Buffer

Guanidine hydrochloride (GuHCl), 6 M, in 50 mM Tris-HCl, pH 7.4

3.6.8. High-salt Tris Buffer

Tris, 1.0 M, in 2.0 M NaCl, 50 mM $CaCl_2$, pH 8.0

3.7. DNA Assay

3.7.1. Proteinase K Buffer

Tris-HCl, 0.05 M, $CaCl_2$, 0.005 M, pH 7.0

Tris-HCl (MW = 121.4)	6.07 g
$CaCl_2$ (MW = 147.0)	0.735 g
UPW	1 L

(i) Add UPW to less than 1 L.

(ii) Adjust the pH to 7.0 with concentrated HCl.

(iii) Add UPW to a final volume of 1 L.

(iv) Store at 4 °C.

3.7.2. Proteinase K Solution

(i) Prepare proteinase K Stock Solution, 5 mg/ml

Proteinase K	100 mg
Proteinase K buffer (See Section 3.7.1)	20 ml

(ii) Dissolve and then freeze 1.0-ml aliquots in 1.5-ml tubes.

(iii) Store at −20 °C

(iv) Prepare a working solution of proteinase K: 125 μg/ml proteinase K in proteinase K buffer (See Section 3.7.1) by adding 500 μl 5 mg/ml proteinase K stock solution in proteinase K buffer to 19.5 ml proteinase K buffer.

Final working solution: 20 ml 125 μg/ml proteinase K (This is a 1/40 dilution).

3.7.3. DNA Standard Solution

Stock Solution: 1 mg/ml calf thymus DNA in UPW.

Prepare approximately 20 ml solution; stir overnight at room temperature, aliquot, and freeze.

Working Solution: 40 μg/ml calf thymus DNA in proteinase K digestion buffer.

Add 40 μl Stock Solution to 960 μl proteinase K digestion buffer.

Prepare Working Solution fresh, as needed.

3.7.4. Hoechst 33258 Dye Buffer

Tris, 0.1 M, in 0.1 M NaCl, pH 8.0

Tris (MW = 121.14)	12.114 g
NaCl (MW = 58.44)	5.844 g

(i) Add UPW to less than 1 L.

(ii) Adjust the pH with concentrated HCl and then add UPW to a final volume of 1 L.

(iii) Filter solution with a 0.22-μm filter.

A higher concentration of Tris and a higher pH than the typical optimum for the Hoechst dye are used in this dye buffer to compensate for the low pH in the papain digestion buffer and the reduced volumes used in the microtiter plate. If the above-listed papain digestion buffer (See Section 3.3.3) is not used, the following dye buffer should be used: 10 mM Tris, 0.1 M NaCl, pH 7.4.

(iv) Store at 4 °C.

Note: This dye buffer is for samples that already contain EDTA in solution. For samples that do not contain EDTA, add 1 mM EDTA to the dye buffer.

3.7.5. Hoechst 33258 Dye Solution

△ ***Safety note.*** HOECHST 33258 DYE IS A MUTAGEN. GLOVES SHOULD BE WORN AT ALL TIMES WHEN HANDLING!

Stock Solution: 1 mg/ml in UPW; stable at 4 °C for 6 months in the dark (wrap the tube in aluminum foil).

Working Solution: 1 μg/ml in Hoechst dye buffer (15 ml/plate; add 15 μl Stock Solution to 15 ml Hoechst dye buffer).

Prepare immediately before use and store in a dark tube/bottle.

4. HARVESTING CARTILAGE AND ISOLATION OF CHONDROCYTES

Protocol 7.1. Harvest of Articular Cartilage Tissue (Day 1)

Reagents and Materials

Sterile

- ❑ Isolation medium (See Section 3.1.1)
- ❑ Saline wash (See Section 3.2)
- ❑ Alcohol, 70%
- ❑ Sample cups, 1 for every 3 hooves
- ❑ Scalpels, #10, #15 blades

Nonsterile

- ❑ Bovine lower front limbs, 3 or more, distal to the carpus, isolated by transecting at the carpus.
- ❑ Paper towels
- ❑ Clamp for holding leg
- ❑ Balance, 100 mg–100 g range

Protocol

A. Bovine Articular Cartilage Tissue–Metacarpophalangeal Joint

One experiment should pool cartilage from 3 or more hooves.

(a) Using sterile technique, fill a sterile sample cup with 50 ml isolation medium.
(b) Cap the cup with the lid before removing it from the tissue culture hood. Prepare 1 sample cup for every 3 hooves being cut.
(c) Preweigh each container with medium alone (without tissue).
(d) Thoroughly clean the leg by running under hot water while scrubbing with a brush.
(e) Use a scalpel with a new #10 blade to remove the skin from the leg:
    i) Cut around the leg just above the hoof.
    ii) Cut lengthwise from the top of the leg to just above the first incision.
    iii) Remove skin.
(f) Thoroughly wash the skinned leg with running water and remove all contaminated material.
(g) Dry the leg with paper towels and place clean dry paper towels on the cut end of the leg to prevent blood from running down the leg.
(h) Place the leg in an appropriate holder.

(i) Place paper towels around the hoof.
(j) Clean the outside of the metacarpophalangeal joint with 70% EtOH.
(k) Using sterile technique (mask and gloves recommended), open the joint with a scalpel (new #10 blade). TO MAINTAIN STERILITY, DO NOT TOUCH THE CARTILAGE INSIDE THE JOINT OR THE SYNOVIAL FLUID WITH THE BLADE THAT HAS TOUCHED THE OUTSIDE OF THE JOINT.
(l) Cut the cartilage from the lower joint, using a scalpel with a #15 blade.
(m) Transfer the cartilage pieces into the sample cup containing medium with sterile forceps.
(n) Cut the cartilage of the upper joint, using a scalpel with a #10 blade, and transfer to sterile cup as in Step (m).
(o) After all possible usable cartilage in the joint is removed, weigh the container with the cartilage.
(p) In the tissue culture hood, remove the medium from each sample cup, using a sterile pipette.
(q) Wash twice, 5 min each wash, with sterile 0.9% NaCl, 100 μg/ml gentamicin (50 ml/wash).
(r) Combine the cartilage from up to 4 hooves in 1 sample cup in preparation for enzyme digestion.

B. Human Articular Cartilage Tissue

(a) If the whole joint is provided, follow a similar procedure to isolate cartilage as *A*.

△ *Safety note.* To minimize the chance for transmission of infectious material, use extra care to avoid injury and splashing. Also, use proper protective gear such as a surgical gown and a facemask with a plastic shield.

(b) In the tissue culture hood, remove the medium from each sample cup with a sterile pipette.
(c) Wash twice, 5 min each wash, with sterile 0.9% NaCl, 100 μg/ml gentamicin (50 ml/wash).
(d) Cut the cartilage into $5 \times 5\text{-mm}^2$ pieces to obtain optimum digestion, keeping the tissue moist at all times.

Protocol 7.2. Isolation of Chondrocytes from Bovine or Human Articular Cartilage (Day 1)

Reagents and Materials

Sterile

- ❑ Culture medium (See Section 3.1.2)
- ❑ Isolation medium (See Section 3.1.1)
- ❑ Sample cup with cartilage pieces
- ❑ Pronase solution (See Section 3.3.1)

- ❑ Collagenase-P solution (See Section 3.3.2)
- ❑ Stirrer bar
- ❑ Cell strainer: 40-μm mesh sieve
- ❑ Centrifuge tube, 50 ml

Nonsterile

- ❑ Trypan Blue, 0.4%
- ❑ Electronic cell counter, or hemocytometer

Pronase Digestion

Protocol

(a) Add the sterile filtered Pronase enzyme digestion solution to the sample cup containing the cartilage pieces.
(b) Insert the stirrer bar and incubate with the cap of the sample cup attached loosely for 1 h, with gentle stirring, in a 37 °C, 5% CO_2 tissue culture incubator.
(c) At the end of the Pronase enzyme digestion incubation period, wash the cartilage 3 times, 2 min each, with isolation medium (30–50 ml per wash).

Collagenase-P Digestion

(d) Add 25 ml 2× Collagenase-P enzyme digestion solution and 25 ml culture medium to the sample cup containing the cartilage pieces, along with a sterile stir bar. The final Collagenase-P concentration is 0.0025%.
(e) Loosely cap the specimen cup and incubate for a maximum of 16 h, with gentle stirring, in a 37 °C, 5% CO_2 tissue culture incubator.
(f) At the end of the Collagenase-P enzyme digestion incubation period, transfer the suspension of released chondrocytes through a 40-μm cell strainer sieve into a 50-ml centrifuge tube.
(g) Centrifuge at 110 *g* for 10 min at 4 °C.
(h) Wash the chondrocytes 3 times as follows:
    i) Discard the supernatant and resuspend the pellet in 25 ml isolation medium.
    ii) Centrifuge at 110 *g* for 10 min at 4 °C.
(i) After the last centrifugation, discard the supernate and resuspend the chondrocytes in 10 ml isolation medium.

Determination of Cell Number

(j) Count the number of chondrocytes in suspension with an electronic cell counter (e.g. Coulter Z1 Particle Counter, upper threshold: 21.54 μm, lower threshold: 8 μm) or hemocytometer.

Determination of Cell Viability

(k) Add 100 μl total chondrocyte cell suspension to 100 μl Trypan Blue stain 0.4%.
(l) Mix and immediately add to a hemocytometer for viewing under the microscope. Dead cells will appear blue because Trypan Blue stain only penetrates the membranes of dead cells. A photographic record should be made.

5. CULTURE OF CHONDROCYTES

5.1. Proliferating Monolayer

Protocol 7.3. Monolayer Culture of Articular Chondrocytes Under Proliferating Conditions

Reagents and Materials

- ❑ Culture medium (See Section 3.1.2)
- ❑ Isolation medium (See Section 3.1.1)
- ❑ Pronase solution, 0.2% (See Section 3.3.1)
- ❑ Collagenase-P solution (See Section 3.3.2)
- ❑ Culture flasks, 150 cm^2
- ❑ Centrifuge tube, 50 ml

Protocol

Initial Seeding

(a) Plate cells at a concentration of 2.5×10^6 cells per 150-cm^2 flask (17,000 cells/cm^2) with a total volume of 20 ml/flask.

(b) Change the medium every other day until cells reach confluence ($\sim$1.5 weeks for first passage, $\sim$1 week for second and subsequent passages).

Passage of Cells after Monolayer Culture

(c) Aspirate media from flasks.

(d) Add equal volumes of standard media and 0.2% Pronase solution (a minimum of 5 ml standard media and 5 ml 0.2% Pronase solution should be used) to each flask. Place in the incubator for 10–30 min or until cells are released from the flask.

(e) Transfer cells from all flasks from the same donor to one 50-ml tube. Rinse each flask with 5–10 ml culture medium, pipetting off any remaining cells. Combine the cells from the rinse with the cells in the 50-ml tube.

(f) Centrifuge at 110 *g* for 10 min at 4 °C.

(g) Aspirate the medium, add 25 ml isolation medium, and centrifuge at 110 *g* for 10 min at 4 °C.

(h) Aspirate the media again, add 12.5 ml culture medium, and transfer cells to a specimen cup containing a sterile stir bar.

(i) Prepare 12.5 ml 2× sterile Collagenase-P solution (0.05% Collagenase-P) in a 50-ml tube and then add to the specimen cups.

(j) Loosely cap the specimen cups and place in the incubator at 37 °C and 5% CO_2 for 1.5 h.

(k) At the end of the Collagenase-P enzyme digestion incubation period, transfer the suspension of released chondrocytes through a 40-μm cell strainer sieve filter into a sterile 50-ml centrifuge tube.

(l) Centrifuge at 110 *g* for 10 min at 4 °C.

(m) Resuspend in growth medium and reseed at 2.5×10^6 cells per 150-cm^2 flask (17,000 cells/cm^2) with a total volume of 20 ml/flask, or proceed to alginate encapsulation (See Protocol 7.4).

5.2. Alginate Beads

Protocol 7.4. Encapsulation of Chondrocytes in Alginate Beads

Reagents and Materials

Sterile

- ❑ Isolation medium (See Section 3.1.1)
- ❑ Culture medium (See Section 3.1.2)
- ❑ Sodium alginate solution (See Section 3.4.1)
- ❑ NaCl solution, 0.9%
- ❑ $CaCl_2$ solution, 102 mM (See Section 3.4.3)
- ❑ Stirrer bar
- ❑ Pipettes, including 50 ml
- ❑ Petri dishes, 10 cm, deep well

Protocol

(a) Prepare a 1.2% alginate solution in sterile 0.9% NaCl; start early, to allow sufficient time for dissolving the alginate. Prepare 25 ml 1.2% alginate solution for cartilage from each hoof (e.g., 150 ml for cartilage from 6 hooves).

(b) Centrifuge the original total chondrocyte cell suspension at 110 *g* for 10 min at 4 °C.

(c) Remove the supernate and add sufficient sterile 1.2% alginate solution to have 4×10^6 chondrocytes/ml of 1.2% alginate solution. Determine the volume of 1.2% alginate solution needed by dividing the total cell number by 4×10^6.

(d) When resuspending the chondrocytes in the 1.2% alginate solution, do not add more than 5 ml alginate solution initially. Mix well and then add the remaining alginate solution needed to obtain the desired chondrocyte concentration.

(e) Add 50 ml sterile 102 mM $CaCl_2$ solution to a sterile sample cup.

(f) Add 7 ml chondrocyte-alginate mixture to 50 ml sterile 102 mM $CaCl_2$ solution. This step is particularly important for human chondrocyte preparations. Up to 12 ml chondrocyte-alginate mixture may be added to 50 ml sterile 102 mM $CaCl_2$ solution:

  i) The $CaCl_2$ solution should be stirred constantly with a sterile stirrer bar.

  ii) Use either a sterile syringe that has a sterile 22-gauge needle attached or a disposable sterile pipette (5 ml or 10 ml) attached to a peristaltic pump.

  iii) Suspend the syringe/pipette above the sample cup at a 45° angle, making sure that the end of the needle/pipette is at a 90° angle with the surface of the $CaCl_2$ solution. A distance of 5 cm from the end of the needle/pipette to the surface of the $CaCl_2$ solution is desired.

iv) Discard the first few drops of the chondrocyte/alginate mixture and then express the chondrocyte/alginate mixture into the $CaCl_2$ solution. The speed should be adjusted so that all of the solution is added in less than 2 min.

(g) Equilibrate the newly formed chondrocyte-containing alginate beads in the $CaCl_2$ solution for 8–10 min, but not longer than 10 min.

(h) Wash the beads 3 times with 25–30 ml sterile 0.9% NaCl, 10 min each wash. Use a Pasteur pipette connected to the vacuum line in the hood to remove the wash solutions.

(i) Wash the beads once for 5 min with 25 ml sterile isolation medium.

(j) Collect the beads with a 50-ml sterile pipette slowly (avoid breaking beads) and transfer to a sterile 10-cm non-tissue culture-grade Petri dish (deep well), where they can be counted and then:

i) Batch culture, 500 beads/one or two 10-cm dishes

or

ii) Dispense into 6-well (150–200 beads/well), 12-well (27 beads/well), or 24-well (9 beads/well) tissue culture plates according to the design of the experiment.

(k) Add culture medium: 22 ml/500 beads; 1.2 ml/27 beads; 400 μl/9 beads.

(l) Incubate in a tissue culture incubator at 37 °C/5% CO_2.

Maintenance of Chondrocytes in Alginate Culture

(m) Change medium daily. Use a 10-ml pipette to remove the medium, with caution to avoid sucking the beads into the pipette. Use 22 ml culture medium per 500 beads.

6. FABRICATION OF CARTILAGINOUS TISSUE BY THE SCAFFOLD-FREE ALGINATE-RECOVERED CHONDROCYTE (ARC) METHOD

Cell-laden cartilaginous tissues can be engineered in vitro and may provide a suitable graft material for cartilage repair. Such tissues are typically formed by multiplication of cells from an appropriate source (e.g., by monolayer culture starting at a low seeding density), differentiation of the cells to the chondrocytic phenotype, and then growth in vitro under controlled conditions. Compared to direct implantation of cells, such engineered tissues would be predicted to shorten the postoperative rehabilitation time because the processes of differentiation and matrix formation have already occurred or begun in vitro. Such tissues have been synthesized primarily with immature chondrocytes or chondroprogenitor cells, in combination with various types of scaffolds.

Chondrogenic cells have often been mixed with or infiltrated into a number of scaffold materials to form cartilaginous tissue. Such materials are typically degradable and synthetic, such as polylactic acid [Dounchis et al., 2000] or polyglycolic

acid [Dunkelman et al., 1995; Vunjak-Novakovic et al., 1999], or natural, such as those derived from or analogous to collagen [Wakitani et al., 1989], hyaluronan (HA) [Robinson et al., 1990; Solchaga et al., 2001], fibrin [Itay et al., 1987; Hendrickson et al., 1994], and alginate-fibrin mixture [Perka et al., 2000]. Other materials, such as agarose, have been used primarily as model systems for analyzing the regulation of tissue formation [Buschmann et al., 1992, 1995; Mauck et al., 2003]

Other methods have been developed to form implants that are composed only of cells and their products. Immature chondrocytes can form cartilaginous tissue when cultured as a monolayer at high density in the absence of a scaffold [Adkisson et al., 2001] or as a multilayer [Yu et al. 1997], a configuration that recapitulates the high density of cells in fetal cartilage [Li et al., 2001]. On the other hand, chondrocytes from adult articular cartilage form only a very small amount of matrix when cultured under the same conditions [Adkisson et al., 2001]. For the production of cartilaginous tissue in vitro, we developed a two-step culture method, termed the alginate-recovered chondrocyte (ARC) method [Masuda et al., 2003].

The ARC method allows formation of cartilaginous tissue from animal or human cells with culture in alginate gel as an intermediate step. The methodology is robust enough to use cells from adults, which are typically difficult to stabilize in a chondrogenic phenotype. The first step of the ARC method consists of culturing chondrocytes encapsulated in alginate gel under conditions that maintain the normal phenotype of chondrocytes and modulate the formation of a CM rich in aggrecan molecules. After 1 week, the alginate gel is solubilized and the cells with their attached CM are recovered and cultured on a porous membrane. Within 1 further week the alginate-recovered chondrocytes become integrated into a cohesive cartilaginous tissue mass containing abundant amounts of aggrecan and type II collagen and minimal amounts of type I collagen [Masuda et al., 2003].

Once there is a sufficient quantity of cell-associated matrix in the cultured alginate beads, the beads can be dissolved and the cells with their cell-associated matrix recovered. For bovine cells, an appropriate preculture period is 7–10 days when cultured in the presence of 20% FBS. For human cells, approximately 2 weeks is effective, when incubation is done in medium supplemented with 200 ng/ml osteogenic protein 1 (OP-1; BMP-7) and 20% FBS.

Protocol 7.5. Release of Chondrocytes from Alginate Beads

Reagents and Materials

Sterile

- ❑ Alginate dissolving buffer (See Section 3.4.2)
- ❑ Saline wash (See Section 3.2)
- ❑ Isolation medium (See Section 3.1.1)
- ❑ Culture medium (See Section 3.1.2)
- ❑ Spatula
- ❑ Centrifuge tube, 50 ml

Protocol

(a) Collect beads in a sterile 50-ml tube with a sterile spatula as follows:
  i) Bovine, collect 300 beads/insert (See Protocol 7.6)
  ii) Human, collect 350 beads/insert
  iii) Porcine, collect 300–350 beads/insert
(b) Add 20 ml alginate dissolving buffer to each tube.
(c) Mix the tube gently. **Do Not Vortex.** Let it sit at room temperature for 30 min.
(d) After the beads have dissolved, centrifuge at 110 *g* for 10 min at 4 °C.
(e) Remove the supernate and wash the cell pellet 2× with saline wash. Centrifuge at 110 *g* for 10 min at 4 °C each time, removing the wash solutions with a Pasteur pipette connected to the vacuum line in the hood. Leave approximately 2 ml after each aspiration.
(f) Wash the cell pellet once with 20 ml isolation medium.
(g) Centrifuge at 110 *g* for 10 min at 4 °C.
(h) Resuspend in culture medium as required.

Protocol 7.6. Culture of ARC Chondrocytes and Associated Matrix in Filter Well Inserts

Reagents and Materials

Sterile

- ❑ Culture medium (See Section 3.1.2)
- ❑ Filter well inserts, 23 mm, 4-μm porosity, polyethylene terephthalate (PET)
- ❑ Multiwell plate, 6 well, deep well
- ❑ Scalpel

Protocol

Seeding

(a) Add 15 ml culture medium to each well of the 6-well deep-well companion plate before positioning the cell culture insert.
(b) Resuspend the cells in 5 ml culture medium.
(c) Pipette the 5 ml cell suspension onto the 0.4-μm inserts in the 6-well plates. (***Note***: inspect inserts for holes before adding the cell suspension.)
(d) The cell suspension should be allowed to settle on the insert for 1 full day without movement before changing the medium.
(e) Change the medium every other day after the first 2 days (15 ml outer well, followed by 5 ml to the insert).
(f) Collect inserts after 2–3 weeks.

Maintaining Cultures of ARC Tissue

(g) Remove medium from both the bottom of the deep-well plate and the filter well insert.

(h) Carefully add complete medium onto the insert. (***Note:*** do not feed dropwise at the beginning of culture; touch the pipette to the wall of the insert and add slowly.)

Recovering ARC Tissue

(i) Once culture is complete, remove each insert from the 6-well plate and place in a Petri dish.

(j) Cut the PET membrane along its circumference with a scalpel and peel away the membrane carefully to release the de novo ARC tissue.

7. CRITERIA FOR EVALUATION OF CARTILAGINOUS TISSUE FORMATION

There are a variety of ways to test whether engineered cartilaginous tissue has a composition, structure, function, and metabolism similar to cartilage and indicative of cells expressing the normal chondrocytic phenotype. Biochemical analyses include the determination of proteoglycan (PG) content, collagen content and type, and cellularity assessed as DNA content. Biomechanical analyses include the determination of compressive and tensile functions. The results of these analyses can be compared to those of normal cartilage, with consideration of the natural changes in the tissue that occurs with growth, aging, and degeneration as well as the heterogeneity of cartilage with depth from the articular surface and location in various joints [Sah, 2002].

7.1. Proteoglycan

Protocol 7.7. Determination of Proteoglycan Content of Chondrocyte Cultures from Filter Well Inserts

Reagents and Materials

Nonsterile

- ❑ Excised insert cultures (See Protocol 7.6 Step (j))
- ❑ Papain enzyme digestion solution, 20 μg/ml (See Section 3.3.3)
- ❑ PG standard stock, 2 mg/ml (See Section 3.5.1)
- ❑ PG dilution buffer (See Section 3.5.2)
- ❑ Boiling water bath
- ❑ Guanidine hydrochloride (See Section 3.5.4)
- ❑ Dimethylmethylene blue (DMMB) dye reagent (See Section 3.5.3)

Protocol

A. Papain Digestion of Sample

Digestion can be performed on all of the samples from one experiment at the same time.

(a) Add an appropriate amount of the 20 μg/ml papain enzyme digestion solution to each sample. The volume required for a 23-mm diameter piece of ARC tissue is at least 3 ml.

(b) Incubate the samples (and blank controls; See Section 3.3.3) overnight at 56–60 °C.
(c) At the completion of digestion, all samples should be boiled for 5 min to inactivate the enzyme.

B. Assay for PG Content

(d) Determine the content of sulfated PG molecules in the papain-digested sample with a modified dimethylmethylene blue (DMMB) dye binding method [Chandrasekhar et al., 1987]. The dynamic range of the DMMB assay is very narrow. Obtaining a pinkish color on PG-dye binding indicates that the dye is already saturated and that dilution to a lower concentration is necessary. For that reason, it is recommended to perform at least 6 dilutions for each sample in a 96-well plate to accurately measure the amount of glycosaminoglycan by the DMMB method.

Standard Dilution Scheme

i) From the 2 mg/ml PG stock solution, prepare a working stock solution of 16 μg/ml PG. This is a dilution factor of 1/125.
ii) A total of 7 dilutions should be performed for the standard, in duplicate, for each plate.
iii) Place standards on the top and bottom of the plate (A2-A9, H2-H9).
iv) Wells A1 and H1 should be left empty for the blank.
v) Pipette 263 μl 16 μg/ml PG standard working stock solution to the first well (A2, H2).
vi) To the remaining wells pipette 75 μl dilution buffer.
vii) Perform serial dilutions of 1/1.4 for the remaining wells by transferring 188 μl with a multichannel pipette.
viii) 188 μl of A2 is transferred to A3 and mixed, then 188 μl of A3 is transferred to A4, etc. A dilution of 188 μl up to 263 μl is a 1/1.4 dilution factor

Final volume in all standard wells after dilution = 75 μl.
Concentration Range of Standard (μg/ml): 16; 11.43; 8.16; 5.83; 4.16; 2.97; 2.12

Note: The working range of the standard is only from 11.43 μg/ml to 2.12 μg/ml. The concentration of 16 μg/ml is a convenient starting dilution by which to obtain the working range and should not be used in calculations because it is off scale.

Sample Dilution Scheme

i) Pipet 150 μl diluted sample into the first well.
ii) To the remaining wells pipet 75 μl dilution buffer.
iii) Perform serial dilutions of 1/2 by transferring 75 μl; 75 μl of B1 is transferred to B2 and mixed, then 75 μl of B2 is transferred to B3, etc. A dilution of 75 μl up to 150 μl is a 1/2 dilution factor, and the final volume in all sample wells after dilution is 75 μl.

Addition of Guanidine Hydrochloride (GuHCl)

It is important to maintain a concentration of 0.24 M GuHCl/well to prevent PG-dye complex precipitation as well as the interference of HA and/or DNA.

i) After dilution of standards and samples is complete, add 25 μl 2.88 M GuHCl to each well.
ii) The final concentration per well is 0.24 M after the addition of DMMB reagent.

Reaction with DMMB

iii) Add 200 μl DMMB reagent to each well with a multichannel pipette.
Blank: (Microtitration wells A1, H1) 275 μl dilution buffer + 25 μl 2.88 M GuHCl
Background: (Microtitration wells A12, H12) 75 μl dilution buffer + 25 μl 2.88 M GuHCl +200 μl DMMB reagent
iv) Put plates on a shaker for 30 s to ensure thorough mixing.
v) Measure the absorbance for each plate at 530 nm and 595 nm

Note: It is important to read the plates as soon as possible after the addition of DMMB because the color intensity decreases over time. If assaying more than one plate, reagent should not be added to all plates at once. Each plate should have its absorbance read immediately after the addition of DMMB.

Once the 530-nm and 595-nm absorbance data are measured, the ratio should be calculated by using software provided with the plate reader or an Excel spreadsheet.

Calculation of Amount of PG in Samples

A plot of absorption against PG standard concentrations yields a linear response between roughly 2.97 μg/ml and 11.43 μg/ml. The equation of this line can be used to determine the amount of PG present in the unknown samples. It is important to correct for the dilution factor when necessary.

7.2. Collagen Content

The content of hydroxyproline can be measured by phenylisothiocyanate (PITC) derivatization and isocratic reverse-phase high-performance liquid chromatography (HPLC) [Chiba et al., 1997] as a measure of collagen molecules. To calculate the amount of collagen content in each sample, the measured hydroxyproline content is multiplied by a conversion factor of 7.25 [Herbage et al., 1977; Pal et al., 1981].

Protocol 7.8. Determination of Collagen Content of Chondrocyte Cultures from Filter Well Inserts

Reagents and Materials

Nonsterile

- ❑ HCl, 12 N

- ❑ TEA drying solution (See Section 3.6.1)
- ❑ Derivatizing solution (See Section 3.6.2)
- ❑ Collagen assay standard tubes: lyophilized L-proline (20 mg/tube)
- ❑ Reconstitution buffer (See Section 3.6.4)
- ❑ Hydroxyproline HPLC eluent A (See Section 3.6.5)
- ❑ Hydroxyproline HPLC eluent B (See Section 3.6.6)
- ❑ Hydroxyproline HPLC standard (See Section 3.6.3)
- ❑ Screw cap tubes, glass (hydrolysis tubes) with caps with PTFE liners
- ❑ Microcentrifuge filter tubes, 0.22-μm pore size, nylon membrane (Corning Costar Spin-X HPLC tubes)
- ❑ Heating block to fit hydrolysis tubes
- ❑ Lyophilizer
- ❑ HPLC
- ❑ HPLC columns: 5-μm C18 precolumn (45 × 4.6 mm) and a 5-μm C18 column (250 × 4.6 mm)

Protocol

A. Sample Preparation

(a) Estimate the volume of sample required that gives 2 μg hydroxyproline (1 or 0.5 μg may be used if necessary). Make sure that the total volume is under 200 μl and then bring up to a final volume of 200 μl by adding dH_2O.
(b) Pipette this into hydrolysis tubes.

B. Sample Hydrolysis

(c) Add 200 μl 12 N HCl/200 μl sample.
(d) Firmly tighten the screw caps and vortex.
(e) Place samples in the heating block at 120 °C for 16–20 h (overnight).
(f) Centrifuge the tubes before uncapping and then lyophilize to dry.

C. Sample Drying

(g) Once the samples are dry, add 20 μl drying solution to each sample. The drying solution neutralizes any excess HCl.
(h) Lyophilize or concentrate the samples to dry.
(i) Repeat this procedure once.

D. Sample Derivitization

(j) Add a 20-μl aliquot of freshly prepared derivatizing solution to each sample, as well as to the proline standards, and vortex.
(k) Leave for 10 min at room temperature in a fume hood for the reaction to occur before lyophilizing or concentrating the samples and standards to dryness.

△ *Safety note.* PITC is very harmful—wear gloves and use in a fume hood.

E. Sample Reconstitution

(l) Dissolve samples in 1 ml reconstitution buffer and centrifuge for 5 min in microcentrifuge filter tubes, 0.22-μm pore size, nylon membrane (Corning Costar Spin-X HPLC tubes).

(m) Dissolve standard in 1 ml reconstitution buffer and centrifuge as above. Final standard concentration = 20 mg/ml.

F. Hydroxyproline Determination with HPLC

Hydroxyproline content is determined with isocratic separation and fluorescence detection at 254λ.

(n) Utilizing a 5-μm C18 precolumn (45 × 4.6 mm) and a 5-μm C18 column (250 × 4.6 mm), elute samples in the following manner.
   i) At start up, equilibrate columns with 100% eluant A (See Section 3.6.5) for 15 min at a flow rate of 1 ml/min.
   ii) Column pressure is typically 2000 psi at 1 ml/min.
   iii) Load samples onto the column (10–100 μl) with an autosampler.
   iv) Elution of the hydroxyproline peak will occur at approximately 9 min with 100% eluant A at a flow rate of 1 ml/min.
   v) At 13 minutes, switch to 100% eluant B at a flow rate of 1.5 ml/min for a duration of 3 min. This wash step serves to strip the column of previous sample residues.
   vi) Finally, reequilibrate the columns with 100% eluant A for 13 min at a flow rate of 1 ml/min.
   vii) Once the initial sample run is complete, proceed to the next sample immediately after the 13-min reequilibration.
   viii) Samples are monitored on an absorbance detector at 254 nm, yielding a hydroxyproline peak at approximately 9 min.
   ix) Hydroxyproline sample content is quantified relative to a known amount of derivatized hydroxyproline standard.
   x) Standards are run after every 10 samples to ensure appropriate elution times and run conditions. A reproducible and linear profile of standard concentrations is achieved by running 20-, 50-, and 100-μl injections alternately. 20 μl injected standard (20 ng/μl) equals 400 ng hydroxyproline.

Identification and content of hydroxyproline are determined by the standard elution time and standard peak area ratios.

Protocol 7.9. Determination of Collagen Type

Reagents and Materials

- ❑ Collagen extraction buffer (See Section 3.6.7)
- ❑ High-salt Tris buffer (See Section 3.6.8)
- ❑ Phosphate-buffered saline (PBS) or Tris-buffered saline (TBS)
- ❑ Laemmli sample buffer (Bio-Rad)
- ❑ Acetic acid, 0.05 M
- ❑ NaOH, 1 N
- ❑ NaCl, crystalline

- ❑ Ammonium sulfate, crystalline
- ❑ Phenol red indicator solution
- ❑ Pepsin stock solution (See Section 3.3.4)
- ❑ Elastase stock solution (See Section 3.3.5)
- ❑ Materials for 7.5% SDS-PAGE
- ❑ Coomassie brilliant blue R-250 stain (CB)
- ❑ 10% acetic acid, 10% methanol solution or Bio-Rad destaining solution (overnight) or 10% acetic acid, 40% methanol solution (1 h) for destaining gel
- ❑ Liquid nitrogen
- ❑ Mortar and pestle or glass homogenizer
- ❑ Centrifuge tubes, 5 ml, high *g*
- ❑ Shaker
- ❑ Electrophoresis gel imager and software

Protocol

A. Collagen Extraction from Native and ARC Cartilage

(a) All of the procedures described here should be performed at 4 °C unless otherwise specifically indicated.
(b) The native cartilage should be pulverized after freezing in liquid nitrogen. The ARC tissue can be minced and homogenized in a glass homogenizer.
(c) Add 10 volumes collagen extraction buffer and extract overnight. This helps to enhance the pepsin digestion by removing the PG.
(d) Centrifuge at 10,600 *g* for 10 min at 4 °C. Remove the supernatant and save the pellet.
(e) Wash the pellet with 3 ml 0.05 M acetic acid by spinning and removing the supernate as described above.
(f) To the pellet, add 10 volumes 0.05 M acetic acid, and then adjust the pH to 2.8–3.0 with 0.05 M acetic acid.
(g) Add 1/10 volume pepsin stock solution.
(h) Digest overnight at 4 °C on a rocker/shaker.
(i) After overnight digestion, add 1/10 volume high-salt Tris buffer.
(j) Adjust pH to 8.0 with 1 N NaOH. Adding a drop of phenol red helps to determine the approximate pH.
(k) Add 1/10 volume pancreatic elastase.
(l) Digest for 30 min at 35 °C.
(m) Centrifuge at 10,600 *g* for 10 min at 4 °C.
(n) Collect the supernate.
(o) Save the pellet for further extraction if needed.
(p) Add crystalline NaCl to 3 M and crystalline ammonium sulfate to 30% and incubate for 30 min at 4 °C.
(q) Centrifuge at 10,600 *g* for 10 min at 4 °C and remove the supernate. The pellet contains the precipitated collagen.
(r) Resuspend the pellet in a low-salt buffer, such as 1 × PBS or 1 × TBS.

B. SDS-PAGE of Sample

(a) Dilute samples 1:2 with Laemmli sample buffer according to the supplier's instructions.
(b) Load the samples on a 7.5% SDS-PAGE gel.
(c) Run at room temperature: 60 V for run through the stacking gel, approximately 15 min; 200 V for run through the separating gel, approximately 30–40 min.
(d) Stain gel for protein
    i) Immediately after the gel run is complete, place the gel in 1× Coomassie brilliant blue R-250 stain (CB).
    ii) Incubate for 30 min to 1 h at room temperature with shaking.
(e) Destain gel
    i) *Overnight:* Use either a 10% acetic acid, 10% methanol solution or the Bio-Rad destaining solution.
    ii) *One hour:* Use a 10% acetic acid, 40% methanol solution.
    iii) Multiple solution changes, at room temperature with shaking, are necessary depending on the length of staining time.
(f) Gel storage: Gels may be stored indefinitely in a 10% acetic acid solution before drying. Acetic acid maintains gel integrity.
(g) Gel imaging:
    i) While wet, the gels can be digitized with a commercially available imager. To keep as a record, dry the gels and scan, using a regular scanner with a transparency unit.
    ii) Each α1 and α2 band can be scanned and quantified with NIH Image software to calculate the ratio of types I and II.

Protocol 7.10. Determination of DNA Content

Reagents and Materials

- ❑ Sodium dodecylsulfate (SDS) 0.02%
- ❑ Alginate dissolving buffer with EDTA (See Section 3.4.2)
- ❑ DNA standard solution, 40 μg/ml (See Section 3.7.3)
- ❑ Hoechst dye solution: 1 μg/ml Hoechst 33258 (See Section 3.7.5)
- ❑ Proteinase K solution (See Section 3.7.2)
- ❑ Proteinase K working solution (See Section 3.7.2)
- ❑ Microplate reader

A. Proteinase K Digestion of Alginate Beads for DNA Analysis

Digestion should be performed on all of the samples from one experiment at the same time.

(a) Add 100 μl 0.02% SDS to 9 alginate beads (do not dissolve beads beforehand!).
(b) Heat at 100 °C for 5 min.
(c) Add 400 μl 125 μg/ml proteinase K working solution (See Section 3.7.2) to each sample.

(d) Incubate overnight at 56–60 °C.
(e) Add 400 μl alginate dissolving buffer with EDTA to each sample.
(f) Shake on the shaker for 3 h at room temperature.
(g) The final volume for all samples is 1 ml.

B. Fluorometric Assay of DNA

(h) Prepare standard dilutions. A total of 10 dilutions should be performed for the standard, in duplicate, for each plate. Place standards on the top and bottom of the plate (A2–A7, H2–H7).
    i) Pipette 200 μl DNA Standard Working Solution (40 μg/ml) to wells A2 and H2.
    ii) Pipette 100 μl papain digestion buffer to wells A3–A7 and H3–H7.
    iii) Perform serial dilutions of $\frac{1}{2}$ by transferring 100 μl from A2 to A3 and mixing, then 100 μl from A3 to A4, etc.

Final Standard Concentrations (μg/ml) are: 40; 20; 10; 5; 2.5; 1.25.

(i) Add sample preparation: 100 μl proteinase K-digested sample/well (rows B–G).
(j) Add 100 μl Hoechst 33258 working-strength dye solution/well.
    i) The dye/sample complex is stable for 2 h, so the fluorescence should be measured within this time.
    ii) Blank: (A1, H1); 100 μl papain buffer + 100 μl Hoechst dye buffer.
    iii) Background: (A12, H12); 100 μl papain buffer + 100 μl 1 μg/ml Hoechst 33258 dye solution.

Read fluorescence on microplate reader at 360 nm excitation and 460 nm emission. Sensitivity: 36% and 40%.

C. Calculation of DNA in Samples

A plot of fluorescence against DNA concentration of the standards yields a linear response between 1.25 μg/ml and 40 μg/ml. The equation of this line can be used to determine the amount of DNA present in the unknown samples.

7.3. Outcome of Tissue Analyses

The addition of certain growth factors, such as osteogenic protein-1 (OP-1; BMP-7), to both steps of culture can significantly enhance the formation of tissue in vitro [Masuda et al., 2003]. With the ARC method and the inclusion of OP-1, cells obtained from a small biopsy of knee cartilage and expanded in monolayer culture can be induced to form cartilaginous tissue within 6 weeks [Masuda et al., 2002].

Variations of the ARC method involve the use of cells with different phenotypic characteristics [Klein et al., 2003]. The use of such cell populations allows formation of tissue with stratification and depth-varying properties, resembling native cartilage. In particular, cells from the superficial zone of cartilage have been used as the source of cells forming the superficial region of engineered tissue. Such cells normally secrete superficial zone protein (SZP) [Schumacher et al., 1994, 1999; Flannery et al., 1999]. SZP appears to have an important role for the mechanical function of articular cartilage, as it is identical or closely related to the

molecule termed lubricin, which imparts lubrication properties to the articular surface [Swann et al., 1985; Jay et al., 1998, 2001; Schmid et al., 2002]. Cartilaginous tissue, engineered to be stratified, also secretes SZP from cells in its surface layer.

7.4. Assessment of Biomechanical Properties

The biomechanical properties of the formed tissue can be determined with compressive [Williamson et al., 2001] and tensile [Williamson et al., 2003] test methods.

Protocol 7.11. Measurement of Compressive Properties of Filter Well Insert Constructs

Reagents and Materials

- ❑ Phosphate-buffered saline (PBS)
- ❑ Calipers
- ❑ Disk punch, 9.6 mm
- ❑ Compression test instrument

(a) Removing the cultured tissue from the insert, as previously described (See Protocol 7.6, Step (j)).

(b) Measure the thickness of the tissue disk in several locations, and take the average to determine the thickness for mechanical testing.

(c) Measure compressive strength:
   i) Punch out a disk that is 9.6 mm in diameter.
   ii) Place the disk into a radially confining well of the mechanical test instrument, filled with PBS, and apply to the top a fluid-saturated porous compression platen.
   iii) Apply compressive displacements to 15%, 30%, and then 45% amplitude, at a strain rate of 0.02%/s. After each compression, allow stress-relaxation to occur until equilibrium is reached (typically $\sim$30 min for a 1-mm-thick sample of engineered cartilage tissue). Record the equilibrium loads.
   iv) Fit the load-displacement data to determine the compressive modulus extrapolated to the free-swelling state, H_{A0} [Kwan et al., 1990; Chen et al., 2001; Williamson et al., 2001].

(d) Measure tensile properties
   i) After removing the cultured tissue from the insert, as described previously (See Protocol 7.6, Step (j)), measure the thickness of the tissue disk in several locations near the center, and take the average to determine the thickness for mechanical testing.
   ii) From the disk, punch out a tapered specimen, using a custom punch.
   iii) Place the specimen into the clamps of a mechanical test instrument, separated by 5 mm, with PBS recirculating over the sample. Limit the clamps so that the tissue is compressed by 30–50%, to enable secure gripping while not tearing the sample.

iv) Apply tensile displacements until a tare load of ~0.02 N is attained. Take this position to be the "zero" state. From there, elongate the sample to 10% and then 20% amplitude, at a strain rate of 0.2%/second. After each extension, allow stress-relaxation to occur until equilibrium is reached (typically ~10 min for a 1-mm-thick sample of engineered cartilage tissue). Record the equilibrium loads.

v) Then apply tensile displacement at a constant extension rate of 2%/s, recording the load data every 0.1 s, until the sample breaks.

vi) Fit the equilibrium load-displacement data to determine the tensile modulus, E_t, and the dynamic load-displacement data to determine the ramp stiffness and strength (maximum stress) [Williamson et al., 2003].

SOURCES OF MATERIALS

Item	*Comment*	*Catalog #*	*Supplier*
Articular cartilage, human adult	More than 5 g for primary cultures, more than 20 mg for monolayer expansion		Clinical collaboration
L-Ascorbic acid	Nonsterile	A4544	Sigma-Aldrich
Bovine lower legs with hooves	Each experiment should use more than two hooves		Obtained from a local slaughterhouse
Calcium chloride		C-7902	Sigma-Aldrich
Calf thymus DNA Type I	Store at 4 °C	D1501	Sigma-Aldrich
Cell culture flask, 150 cm^2,	Canted neck, plug-seal cap	355000	BD Biosciences
Cell strainer, 40 μm		2340	BD Biosciences
Centrifuge tubes, 50 ml		2070	BD Biosciences
Collagenase-P, *Clostridium histolyticum,*	Nonsterile; store at −20 °C	1213873	Boehringer Mannheim (Roche Diagnostics)
Costar Spin-X HPLC tubes		8161	Corning
L-Cysteine hydrochloride hydrate	For papain buffer activation; store at 4 °C	C-7880	Sigma-Aldrich
1,9 Dimethylmethylene blue		03610	Polysciences, Inc
DMEM-F-12, 50-50 mix without L-glutamine	Sterile	Fisher Cat. # MT-15-090-CM	Mediatech, Inc
Elastase	Pancreatic, from porcine pancreas	E6883	Sigma-Aldrich
Fetal bovine serum (FBS) defined	Sterile	SH30070.03	Hyclone

Item	*Comment*	*Catalog #*	*Supplier*
Filter well inserts (polyethylene terephthalate (PET) membrane)	Transparent, 0.4-μm pore size, 23-mm effective diameter, 4.2-cm^2 effective growth area of membrane	353090	BD Biosciences
Filter well inserts, 12 well	0.4-μm pore size, 10.5-mm effective diameter, 0.9-cm^2 effective growth area of membrane	353180	BD Biosciences
Formic acid		F0507	Sigma-Aldrich
Gentamicin (50 mg/ml)	Sterile	15750-029	Invitrogen
L-Glutamine (29.2 mg/ml)	Sterile	Fisher Cat. # MT-25005-CI	Mediatech, Inc
Guanidine hydrochloride, 8 M		24115	Pierce Biotechnology, Inc
Hoechst 33258 dye	Store at 4 °C	09460	Polysciences
Human articular cartilage, adult	More than 5 g for primary cultures, more than 20 mg for monolayer expansion		Clinical collaboration
Laemmli sample buffer		161-0737	Bio-Rad
Microtitration plates, 96 well	Cytoplates	CFCPN9650	Applied Biosystems
Multiwell plates, 6 well,	For cell culture inserts	353502	BD Biosciences
Multiwell plates, 12 well	For cell culture inserts	353503	BD Biosciences
Multiwell plates, 6 well,	Deep-well	355467	BD Biosciences
NaCl, 0.9%	Sterile	2F7124	Baxter
Osteogenic protein 1 (OP-1; BMP-7)		354-BP-010	R&D systems
Papain	Store at 4 °C	P-3125	Sigma-Aldrich
Pepsin	Porcine gastric mucosa	P1143	Sigma-Aldrich
Phenylisothiocyanate	(PITC: Edman's Reagent)	26922	Pierce
Polyacrylamide gels:	Ready gel Tris-HCl Gel, 7.5% resolving gel, 4% stacking gel, 10 well	161-1154	BioRad
Pronase protease, *Streptomyces griseus,*	Nonsterile; store at 4 °C	53702	Calbiochem
Proteinase K		P-6556	Sigma-Aldrich
Scalpel holders and #10 and #15 scalpel blades			VWR
Sodium alginate	NE/EP grade for pharmaceutical use	Keltone® LV-(HM)	ISP Alginate
Sodium citrate	Tissue culture grade	BP327500	Fisher Scientific
Sodium formate		S648	Fisher Scientific

Item	*Comment*	*Catalog #*	*Supplier*
Spatula		3004	Corning
Specimen container, 100 ml		25384-078	VWR
Steriflip-GP 50-ml tube, 0.22 μm		SCGP00525	Millipore
Steritop-GV filter unit		SCGV S01 RE	Millipore
Trypan Blue 0.4%		15250-061	Invitrogen
Triethylamine		04884-100	Fisher Scientific

REFERENCES

Adkisson, H.D., Gillis, M.P., et al. (2001) In vitro generation of scaffold independent neocartilage. *Clin. Orthop.* 391S: 280–294.

Athanasiou, K.A., Fischer, R., et al. (1995) Effects of excimer laser on healing of articular cartilage in rabbits. *J. Orthop. Res.* 13: 483–494.

Aubin, P.P., Cheah, H.K., et al. (2001) Long-term followup of fresh femoral osteochondral allografts for posttraumatic knee defects. *Clin. Orthop.* 391S: 318–327.

Benya, P.D., and Shaffer, J.D. (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* 30: 215–224.

Bouwmeester, P., Kuijer, R., et al. (1999) Histological and biochemical evaluation of perichondrial transplants in human articular cartilage defects. *J. Orthop. Res.* 17: 843–849.

Brittberg, M., Lindahl, A., et al. (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N. Engl. J. Med.* 331: 889–895.

Buckwalter, J.A., and Mankin, H.J. (1997). Articular cartilage. Part II: degeneration and osteoarthrosis, repair, regeneration, and transplantation. *J. Bone Joint Surg. Am.* 79-A: 612–632.

Bugbee, W.D., and Convery, F.R. (1999) Osteochondral allograft transplantation. *Clin. Sports Med.* 18: 67–75.

Buschmann, M.D., Gluzband, Y.A., et al. (1995) Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. *J. Cell Sci.* 108: 1497–1508.

Buschmann, M.D., Gluzband, Y.A., et al. (1992) Chondrocytes in agarose culture synthesize a mechanically functional extracellular matrix. *J. Orthop. Res.* 10: 745–758.

Chandrasekhar, S., Esterman, M.A., et al. (1987) Microdetermination of proteoglycans and glycosaminoglycans in the presence of guanidine hydrochloride. *Anal. Biochem.* 161: 103–108.

Chen, A.C., Bae, W.C., et al. (2001) Depth- and strain-dependent mechanical and electromechanical properties of full-thickness bovine articular cartilage in confined compression. *J. Biomech.* 34: 1–12.

Chiba, K., Andersson, G.B., et al. (1997) Metabolism of the extracellular matrix formed by intervertebral disc cells cultured in alginate. *Spine* 22(24): 2885–2893.

Dounchis, J., Harwood, F.L., et al. (2000) Cartilage repair with autogenic perichondrium cell and polylactic acid grafts. *Clin. Orthop.* 377: 248–264.

Dunkelman, N.S., Zimber, M.P., et al. (1995) Cartilage production by rabbit articular chondrocytes on polyglycolic acid scaffolds in a closed bioreactor system. *Biotechnol. Bioeng.* 46: 299–305.

Eyre, D.R., Wu, J.J., et al. (1992) Cartilage-specific collagens: structural studies. In Kuettner, K.E., Schleyerbach, R., Peyron, J.G., Hascall, V.C., eds., *Articular Cartilage and Osteoarthritis*. New York, Raven Press, pp. 119–131.

Ficat, R.P., Ficat, C., et al. (1979) Spongialization: a new treatment for diseased patellae. *Clin. Orthop.* 144: 74–83.

Flannery, C.R., Hughes, C.E., et al. (1999). Articular cartilage superficial zone protein (SZP) is homologous to megakaryocyte stimulating factor precursor and is a multifunctional proteoglycan with potential growth-promoting, cytoprotective, and lubricating properties in cartilage metabolism. *Biochem. Biophys. Res. Commun.* 254(3): 535–541.

Hangody, L., Feczko, P., et al. (2001) Mosaicplasty for the treatment of articular defects of the knee and ankle. *Clin. Orthop.* 391S: 328–336.
Häuselmann, H.J., Aydelotte, M.B., et al. (1992) Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. *Matrix* 12: 116–129.
Häuselmann, H.J., Fernandes, R.J., et al. (1994) Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. *J. Cell Sci.* 107: 17–27.
Hendrickson, D.A., Nixon, A.J., et al. (1994) Chondrocyte-fibrin matrix transplants for resurfacing extensive articular cartilage defects. *J. Orthop. Res.* 12: 485–497.
Herbage, D., Bouillet, J., et al. (1977) Biochemical and physicochemical characterization of pepsin-solubilized type-II collagen from bovine articular cartilage. *Biochem, J* 161: 303–312.
Hjertquist, S.O., and Lemperg, R. (1971) Histological, autoradiographic and microchemical studies of spontaneously healing osteochondral articular defects in adult rabbits. *Calcif. Tiss. Res.* 8: 54–72.
Itay, S., Abramovici, A., et al. (1987) Use of cultured embryonal chick epiphyseal chondrocytes as grafts for defects in chick articular cartilage. *Clin. Orthop.* 220: 284–303.
Jay, G.D., Haberstroh, K., et al. (1998) Comparison of the boundary-lubricating ability of bovine synovial fluid, lubricin, and Healon. *J. Biomed. Mater. Res.* 40: 414–418.
Jay, G.D., Tantravahi, U., et al. (2001) Homology of lubricin and superficial zone protein (SZP): products of megakaryocyte stimulating factor (MSF) gene expression by human synovial fibroblasts and articular chondrocytes localized to chromosome 1q25. *J. Orthop. Res.* 19: 677–687.
Johnson, L.L. (2001) Arthroscopic abrasion arthroplasty: a review. *Clin. Orthop.* 391S: 306–317.
Klein, T.J., Schumacher, B.L., et al. (2003) Tissue engineering of articular cartilage with stratification using chondrocyte subpopulations. *Osteoarthritis Cartilage* 11: 595–602.
Kwan, M.K., Lai, W.M., et al. (1990) A finite deformation theory for cartilage and other soft hydrated connective tissues—I. equilibrium results. *J. Biomech.* 23: 145–155.
Li, K.W., Williamson, A.K., et al. (2001) Growth responses of cartilage to static and dynamic compression. *Clin. Orthop.* 391S: 34–48.
Mankin, H.J. (1982) The response of articular cartilage to mechanical injury. *J. Bone Joint Surg. Am.* 64-A: 460–466.
Masuda, K., Miyazaki, T., et al. (2002) Human tissue engineered cartilage by the alginate-recovered-chondrocyte method after an expansion in monolayer. *Trans. Orthop. Res. Soc.* 27: 467.
Masuda, K., Sah, R.L., et al. (2003) A novel two-step method for the formation of tissue engineered cartilage: the alginate-recovered-chondrocyte (ARC) method. *J. Orthop. Res.* 21: 139–148.
Mauck, R.L., Nicoll, S.B., et al. (2003) Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. *Tissue Eng.* 9(4): 597–611.
Mok, S.S., Masuda, K., et al. (1994) Aggrecan synthesized by mature bovine chondrocytes suspended in alginate. Identification of two distinct metabolic matrix pools. *J. Biol. Chem.* 269(52): 33021–33027.
Muller, B., and Kohn, D. (1999) Indikation und durchführung der knorpel-knochen-anbohrung nach Pridie. *Orthopade* 28(1): 4–10.
O'Driscoll, S.W., and Fitzsimmons, J.S. (2001) The role of periosteum in cartilage repair. *Clin. Orthop.* 391S: 190–207.
Pal, S., Tang, L.-H., et al. (1981) Structural changes during development in bovine fetal epiphyseal cartilage. *Collagen Relat. Res.* 1: 151–76.
Perka, C., Spitzer, R.S., et al. (2000) Matrix-mixed culture: new methodology for chondrocyte culture and preparation of cartilage transplants. *J. Biomed. Mater. Res.* 49(3): 305–411.
Petit, B., Masuda, K., et al. (1996) Characterization of crosslinked collagens synthesized by mature articular chondrocytes cultured in alginate beads—comparison of two distinct matrix compartments. *Exp. Cell Res.* 225: 151–161.
Robinson, D., Halperin, N., et al. (1990) Regenerating hyaline cartilage in articular defects of old chickens using implants of embryonal chick chondrocytes embedded in a new natural delivery substance. *Calcif. Tissue Int.* 46: 246–253.
Sah, R.L. (2002) The biomechanical faces of articular cartilage. in Kuettner, K.E., and Hascall, V.C., eds., *The Many Faces of Osteoarthritis*. New York, Raven Press pp. 409–422.

Sah, R.L., Klein, T.J., Schmidt, T.A., Albrecht, D.R., Bae, W.C., Nugent, G.E., McGowan, K.B., Temple, M.M., Jadin, K.D., Schumacher, B.L., Chen, A.C., Sandy, J.D. (2005): Articular cartilage repair, regeneration, and replacement. In: *Arthritis and Allied Conditions: A Textbook of Rheumatology*, ed by WJ Koopman, Lippincott Williams & Wilkins, Philadelphia, pp. 2277–2301.

Schmid, T.M., Su, J.-L., et al. (2002) Superficial zone protein (SZP) is an Abundant glycoprotein in human synovial fluid with lubricating properties. In Kuettner, K.E., and Hascall, V.C., eds., *The Many Faces of Osteoarthritis*. New York, Raven Press, pp. 159–161.

Schumacher, B.L., Block, J.A., et al. (1994) A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. *Arch. Biochem. Biophys.* 311: 144–152.

Schumacher, B.L., Hughes, C.E., et al. (1999). Immunodetection and partial cDNA sequence of the proteoglycan, superficial zone protein, synthesized by cells lining synovial joints. *J. Orthop. Res.* 17: 110–120.

Shapiro, F., Koido, S., et al. (1993) Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. *J. Bone Joint Surg. Am.* 75-A: 532–553.

Solchaga, L.A., Goldberg, V.M., et al. (2001) Cartilage regeneration using principles of tissue engineering. *Clin. Orthop.* 391S: 161–170.

Steadman, J.R., Rodkey, W.G., et al. (2001) Microfracture: surgical technique and rehabilitation to treat chondral defects. *Clin. Orthop.* 391S: 362–369.

Swann, D.A., Silver, F.H., et al. (1985) The molecular structure and lubricating activity of lubricin isolated from bovine and human synovial fluids. *Biochem. J.* 225: 195–201.

Vunjak-Novakovic, G., Martin, I., et al. (1999) Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage. *J. Orthop. Res.* 17: 130–139.

Wakitani, S., Kimura, T., et al. (1989) Repair of rabbit articular surfaces with allograft chondrocytes embedded in collagen gel. *J. Bone Joint Surg. Br.* 71-B: 74–80.

Wei, X., and Messner, K. (1999) Maturation-dependent durability of spontaneous cartilage repair in rabbit knee joint. *J. Biomed. Mater. Res.* 46: 539–548.

Wei, X., Reaseanen, T., et al. (1998) Maturation-related compressive properties of rabbit knee articular cartilage and volume fraction of subchondral tissue. *Osteoarthritis Cartilage* 6: 400–409.

Williamson, A.K., Chen, A.C., et al. (2003) Tensile mechanical properties of bovine articular cartilage: variations with growth and relationships to collagen network components. *J. Orthop. Res.* 21: 872–880.

Williamson, A.K., Chen, A.C., et al. (2001) Compressive properties and function-composition relationships of developing bovine articular cartilage. *J. Orthop. Res.* 19: 1113–1121.

Yu, H., Grynpas, M., et al. (1997) Composition of cartilaginous tissue with mineralized and non-mineralized zones formed in vitro. *Biomaterials* 18: 1425–1431.