# 4

# Cell Sources for Cartilage Tissue Engineering

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## 1. INTRODUCTION

There are many sites of permanent cartilage within the body. However, because cartilage has specific and distinct functions depending on its location, no two cartilages are the same. Each cartilage has a specific extracellular matrix that is produced by cells termed chondrocytes, which are defined by their production of type II collagen, the major collagen of most cartilage. However, there are differences between chondrocytes, both within and among different cartilaginous tissues. All cartilage extracellular matrices have common constituent molecules, but they are present in different proportions, with some molecules unique to certain types of cartilage. Furthermore, each cartilage has a distinct matrix organization. For these reasons, it is argued that any project for cartilage tissue engineering should include a consideration of the specific type of cartilage one is seeking to repair or regenerate. As a caveat to that statement, it is noted that it is presently unclear how specific any tissue-engineered cartilage needs to be before its implantation, because there are few data to date indicating what form of initial cartilage implant is acceptable, given that the implant may remodel in vivo into the desired cartilage type.

Classically, cartilage is divided into three types: hyaline, elastic, and fibrocartilage. However, for any given type, cartilage differs with site, age, and species. The interest in cartilage tissue engineering is due to the fact that many cartilage types have poor intrinsic regenerative capabilities after injury. Furthermore, degenerative diseases, such as osteoarthritis, cause loss of cartilage from many sites in the body, for which there are presently few biological repair or regenerative therapies. Tissue engineering offers the possibility to replace or repair the lost cartilage. A basic requirement for this goal is consideration of the possible cell types that could

be used for cartilage tissue engineering. Chondrocytes and cells with chondrogenic differentiation potential from embryonic and postnatal sources are currently being used for cartilaginous tissue repair and regeneration studies. This chapter discusses possible cell sources, the isolation of cells from different tissues, the options for their culture in vitro, and the methods and assays of their chondrogenic capacity.

## 2. PREPARATION OF MEDIA AND REAGENTS

### 2.1. Dissection Medium

Hanks' balanced salt solution (HBSS) supplemented with concentrates, penicillin (10,000 units/ml)-streptomycin (10 mg/ml) and Fungizone™ 250 μg/ml

For 500 ml of dissection medium:

1. Add 10 ml penicillin/streptomycin and 5 ml Fungizone to 500 ml HBSS to give final concentrations of 200 U/ml penicillin, 200 μg/ml streptomycin and 5 μg/ml Fungizone.
2. Cool on ice before use.

### 2.2. Predigestion Medium for Human Articular Cartilage

HBSS with penicillin/streptomycin, Fungizone and Pronase

For 100 ml of digestion medium, sufficient for 10 g of cartilage:

(i) Add 750 mg pronase to 96 ml HBSS.

(ii) Warm the solution in a 37 °C water bath with occasional agitation to dissolve the pronase.

(iii) Filter the solution through a 0.22 μm filter.

(iv) Add 2 ml penicillin/streptomycin and 1 ml Fungizone.

### 2.3. Digestion Medium for Human Articular Cartilage

Collagenase A in Opti-MEM 1 reduced-serum medium supplemented with fetal bovine serum, penicillin/streptomycin, and Fungizone. Opti-MEM 1 is a modification of Eagle's minimum essential medium and is supplemented with insulin, transferrin, and selenous acid (ITS). The exact constitution is proprietary information. Alternative ITS supplements are available for addition to media.

For 100 ml of digestion medium, sufficient for 10 g of cartilage:

(i) Add 100 mg of collagenase A to 94 ml Opti-MEM.

(ii) Warm the solution in a 37 °C water bath with occasional agitation to dissolve the collagenase.

(iii) Sterilize by vacuum filtration through a 0.22-μm filter.

(iv) Add 2 ml penicillin/streptomycin, 1 ml Fungizone, and 2 ml sterile FBS.

### 2.4. Digestion Medium for Bovine Articular Cartilage

Collagenase type II in Opti-MEM 1 reduced-serum medium supplemented with fetal bovine serum, penicillin/streptomycin, and Fungizone

For 100 ml of digestion medium, sufficient for 10 g of cartilage:

(i) Add 100 mg of collagenase type II to 94 ml Opti-MEM.

(ii) Warm the solution in a 37 °C water bath with occasional agitation to dissolve the collagenase.

(iii) Add 2 ml FBS.

(iv) Sterilize by vacuum filtration through a 0.22 μm filter.

(v) Add 2 ml penicillin/streptomycin and 2 ml Fungizone.

### 2.5. Ascorbic Acid Stock (5 mg/ml)

L-Ascorbic acid phosphate in ultrapure water (UPW)

For 20 ml of ascorbic acid stock:

(i) Add 100 mg L-ascorbic acid phosphate powder to 20 ml distilled, deionized water.

(ii) Agitate intermittently until the powder is dissolved.

(iii) Sterilize by vacuum filtration through a 0.22 μm filter.

### 2.6. Differentiation Medium

Opti-MEM 1 reduced-serum medium supplemented with ascorbic acid stock (See Section 2.5), penicillin/streptomycin, and Fungizone

For 515.45 ml of primary differentiation medium, to 500 ml of Opti-MEM 1:

(i) Add 5.15 ml penicillin/streptomycin and 5.15 ml Fungizone concentrates as in Section 2.1.

(ii) Add 5.15 ml ascorbic acid stock.

### 2.7. Growth Medium

High-glucose DMEM, containing pyruvate and glutamate, 1:1 with Ham's F-12 nutrient medium, and supplemented with fetal bovine serum, ascorbic acid, penicillin-streptomycin, and Fungizone

For 500 ml of growth medium:

(i) Combine 217.5 ml DMEM and 217.5 ml Ham's F-12 medium.

(ii) Add 50 ml fetal bovine serum.

(iii) Add 5 ml penicillin/streptomycin and 5 ml Fungizone concentrates as in Section 2.1.

(iv) Add 5 ml ascorbic acid stock.

(v) Sterilize by vacuum filtration through a 0.22 μm filter.

### 2.8. Alginate Solution (1.2% Alginate in 150 mM NaCl)

Low-viscosity alginate and sodium chloride in UPW
For 100 ml of alginate solution:

(i) Dissolve 0.87 g sodium chloride in 90 ml water.

(ii) Sterilize by vacuum filtration through a 0.22 μm filter.

(iii) Add 1.2 g sterile, low-viscosity alginate and dissolve.

(iv) Bring to a final volume of 100 ml with sterile water.

### 2.9. $CaCl_2$, 102 mM

Calcium chloride in UPW
For 500 ml of $CaCl_2$ solution:

(i) Dissolve 5.66 g $CaCl_2$ in 450 ml UPW.

(ii) Bring final volume to 500 ml.

(iii) Sterilize by vacuum filtration through a 0.22 μm filter.

### 2.10. Sterile Saline, 0.9%

Sodium chloride in UPW
For 500 ml of sterile saline solution:

(i) Dissolve 4.50 g NaCl in 450 ml UPW.

(ii) Bring final volume to 500 ml.

(iii) Sterilize by vacuum filtration through a 0.22 μm filter.

### 2.11. Alginate Depolymerization Solution

Sodium citrate, 55 mM, sodium chloride, 0.15 M, pH 6.8 in UPW
For 500 ml of alginate depolymerization solution:

(i) Dissolve 8.09 g sodium citrate and 4.35 g sodium chloride in 450 ml UPW.

(ii) Adjust pH to 6.8 with hydrochloric acid.

(iii) Bring volume to 500 ml.

(iv) Sterilize by vacuum filtration through a 0.22 μm filter.

### 2.12. L-Ascorbate-2-Phosphate Stock

Ascorbic acid 2-phosphate, Mg salt in Tyrode's salt solution
For 10 ml of stock:

(i) Weigh out 40.0 mg ascorbic acid 2-phosphate Mg salt.

(ii) Add 10 ml Tyrode's salt solution.

(iii) Use a 0.22 μm filter to sterilize.

(iv) Store 1- to 2-ml aliquots in sterile cryotubes at −20 °C. This solution is stable for up to 30 days at 4 °C and for 1 year at −20 °C.

### 2.13. Dexamethasone ($10^{-3}$ M) Stock

Dexamethasone in ethanol
For 10 ml of stock:

(i) To 3.92 mg dexamethasone add 10 ml 100% ethanol

(ii) Use a 0.22 μm filter to sterilize.

(iii) Store at −20 °C for up to one year.

(iv) Make a $1 \times 10^{-5}$ M working solution by 1/100 dilution of $1 \times 10^{-3}$ M stock in DMEM.

(v) Aliquot (1–2 ml in sterile cryotubes) and store at −20 °C. This solution is stable for up to 30 days at 4 °C and for 1 year at −20 °C.

### 2.14. Transforming Growth Factor-β1 (TGF-β1) Stock

TGF-β1 with bovine serum albumin (BSA) in dilute HCl
For a 10 ng/ml solution:

(i) Prepare buffered solvent by combining 12.5 mg BSA, 12 ml UPW, and 0.5 ml 0.1 N HCl.

(ii) Filter to sterilize.

(iii) Reconstitute 10 μg TGF-β1 with 10 ml prepared buffer.

(iv) Make aliquots of appropriate volumes and store at −70 °C.

### 2.15. Basal Medium

Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, 4 mM glutamine, and supplemented with penicillin/streptomycin, ITS + Premix, and 100 mM sodium pyruvate.

For 500 ml of basal medium, to DMEM-high glucose (475 ml) add:

(i) L-Glutamine (200 mM or 29.2 g/l), 10 ml.

(ii) Penicillin $1 \times 10^4$ U/ml and streptomycin 10 mg/ml, 5 ml.

(iii) ITS + Premix, 5 ml.

(iv) Sodium pyruvate 100 mM, 5 ml.

## 3. ARTICULAR CHONDROCYTES FOR CARTILAGE TISSUE ENGINEERING

### 3.1. Human Articular Cartilage

The predominant focus of cartilage tissue engineering has been on regeneration or repair of articular cartilage defects. Therefore, this section is limited to protocols addressing articular chondrocyte isolation and culture. It should be noted that several other cartilaginous structures have been considered as sources of chondrocytes for tissue engineering purposes, including auricular cartilage [Mandl et al., 2002] and nasal septum [Homicz et al., 2003; Kafienah et al., 2002; Rotter et al., 2002]. These chondrocyte sources offer possibilities for use in reconstructive procedures [Chang et al., 2001].

In light of issues related to potential disease transmission and immunological incompatibilities, the current clinical approaches to human articular cartilage tissue engineering involve collection and ex vivo expansion of autogenous chondrocytes. In this approach, approximately 300–450 mg of healthy cartilage is arthroscopically removed from a non-weight-bearing area of the joint, usually from the abaxial surface of the femoral trochlear ridges [Brittberg et al., 1994]. For research purposes, human articular cartilage can also be obtained from tissues discarded during total joint arthroplasty (TJA) and limb amputation procedures and from organ donor sources [Reginato et al., 1994; Stokes et al., 2002]. TJA specimens can generally be obtained within hours of removal from the patient but are overtly pathological material and are usually acquired from older individuals. In contrast, organ donor sources provide healthy specimens from a broader age range. It should be noted that allogeneic chondrocytes have been used in a number of in vivo studies in animal models of articular cartilage repair. Although the outcomes of these studies vary considerably, immunological reactivity does not appear to be a significant factor in the host response to chondrocyte transplantation. In the future, allogeneic sources of articular chondrocytes may prove to be clinically acceptable for human procedures.

Protocol 4.1 details the steps involved in isolating articular chondrocytes from a TJA tissue specimen. The protocol is equally relevant to collection from other sources, such as postmortem, amputation, and organ donor specimens.

## Protocol 4.1. Isolation of Human Articular Cartilage Chondrocytes

***Reagents and Materials***

*Sterile*

- ❑ Dissection medium (See Section 2.1)
- ❑ Predigestion medium for human articular cartilage (See Section 2.2)
- ❑ Digestion medium for human articular cartilage (See Section 2.3)
- ❑ Scalpel with #10 or #15 blade
- ❑ Plastic centrifuge tube (e.g., Falcon), 50 ml, or small Erlenmeyer flask
- ❑ Steriflip filters
- ❑ Bottle filters
- ❑ Filters, 40 μm

*Nonsterile*

- ❑ Trypan Blue
- ❑ Ice bath

***Protocol***

(a) Weigh the container (such as a 50-ml Falcon tube or small Erlenmeyer flask) that will be used to hold the cartilage.

(b) Excise the cartilage from the joint surface in 2- to 3-mm-thick blocks, using a #10 or #15 scalpel blade. Areas of unacceptable pathology should be excluded from the collection.

(c) Place the explants into dissection medium and hold on ice until completion of the cartilage dissection.

(d) Wash the explants several times in dissection medium. Swirl the explants in 10–15 volumes of dissection medium, then aspirate the liquid as the explants settle to the bottom of the container. Repeat this until the supernatant remains clear after agitation.

(e) After completing the wash steps, remove as much liquid as possible and re-weigh the container. The difference between the first and second weights gives a reasonable estimate of the weight of cartilage collected. In practice, between 3.0 and 6.0 g of cartilage can be collected from a single TJA specimen, depending primarily on the degree of pathology ($4.86 \pm 1.97$ g, $n = 20$).

(f) Incubate the explants in predigestion medium for human articular cartilage (1 g/10 ml) in a shaking incubator at 37 °C for 1 h.

(g) Rinse the explants in dissection medium.

(h) Add the digestion medium for human articular cartilage (1 g/10 ml).

(i) Replace the flask in a shaking incubator at 37 °C overnight.

(j) After overnight digestion, filter the medium through a 40 μm filter.

(k) Pellet the isolated cells by centrifugation at 250 *g* for 10 min.

(l) Aspirate the supernatant and resuspend the cells in 10 volumes of Ham's F-12 medium.

(m) After 2–3 rounds of centrifugation and resuspension, determine the cell number with a hemocytometer and test for viability by Trypan Blue exclusion.

Approximately $2-5 \times 10^6$ viable cells can be isolated per gram of cartilage ($3.48 \pm 1.50 \times 10^6$ cells/g, $n = 20$), varying with donor age and the degree of pathology.

Collection of articular cartilage from human tissues should be carried out with all appropriate personal protection required for handling biohazardous material. Similarly, all plasticware, blades, and liquids used in the procedure should be handled appropriately as biohazardous waste.

For a shorter (3–6 h) digestion, the collagenase concentration can be increased up to 0.40%. The FBS can be omitted with shorter digestion times. Note that with higher collagenase concentrations, filtration can be difficult.

### 3.2. Articular Chondrocytes from Other Species

Several animal species are routinely used for articular cartilage and chondrocyte research: rabbits, pigs, goats, dogs, horses, and cattle. Sufficient quantities of articular cartilage can be collected from the joints of these species for in vitro experiments. It is possible to isolate up to $1 \times 10^8$ articular chondrocytes from extensive collections from equine or bovine limbs. These sources are not compromised by preexisting pathology, delays in acquisition, or potential biohazardous risks. Of particular relevance to tissue engineering research, articular cartilage defect models for in vivo analyses of engineered constructs are feasible in these species.

Protocol 4.2 details the steps involved in isolating articular chondrocytes from young adult (15–18 month old) bovine metacarpophalangeal joints. This source is commonly obtained from abattoirs, because the lower limbs are removed from bovine carcasses early in the butchering process. The protocol is equally applicable to other experimental species, such as dog, horse, goat, and rabbit.

## Protocol 4.2. Isolation of Articular Chondrocytes from Other Species

#### *Reagents and Materials*

*Sterile*

- ❑ Dissection medium (See Section 2.1)
- ❑ Trypsin-EDTA: Trypsin, 0.25%, EDTA, 1 mM in phosphate-buffered saline lacking $Ca^{2+}$ and $Mg^{2+}$ (PBSA), supplemented with penicillin 200 U/ml and streptomycin 200 μg/ml
- ❑ Digestion medium for bovine articular cartilage (See Section 2.4)
- ❑ Erlenmeyer flasks, 500 ml and 50 ml
- ❑ Scalpel with #10 or #15 blade
- ❑ Filters, 40 μm
- ❑ Petri dish, 10 cm

*Nonsterile*

- ❑ Trypan Blue
- ❑ Ice bath

***Protocol***

(a) Weigh the container (500 ml Erlenmeyer flask) to be used to hold the sample.
(b) Excise the cartilage from the joint surface with a #10 or #15 scalpel blade and place the explants into dissection medium on ice. Approximately 7 g of cartilage can be excised from the articular metacarpophalangeal joint surfaces of a yearling (15–18 month old) steer.
(c) After excision of the articular cartilage, wash the explants several times in dissection medium. Repeat the washes until the supernatant remains clear after agitation.
(d) At the completion of the wash steps, remove as much liquid as possible and reweigh the container. The difference between the first and second weights gives a reasonable estimate of the weight of cartilage collected.
(e) Transfer the cartilage into a sterile 10 cm Petri dish and immerse in dissection medium.
(f) Dice the samples into approximately 2 mm-thick slices.
(g) Transfer the diced cartilage into an Erlenmeyer flask.
(h) Predigest the explants in trypsin-EDTA (10 ml/g) containing penicillin-streptomycin in a shaking incubator at 37 °C for 1 h.
(i) Rinse the explants in dissection medium, then add the digestion medium for bovine articular cartilage. Replace the flask in a shaking incubator at 37 °C overnight.
(j) After overnight digestion, filter the digestion medium through 40 μm filters.
(k) Pellet the isolated cells by centrifugation at 250 *g* for 10 min.
(l) Aspirate the supernatant and resuspend the cells in 10 volumes of Ham's F-12 medium.
(m) After 2–3 rounds of centrifugation and resuspension, cell number can be determined with a hemocytometer and assessed for viability by Trypan Blue exclusion. Approximately $1 \times 10^6$ chondrocytes ($9.69 \times 10^5 \pm 1.85 \times 10^5$, $n = 8$) can be isolated from each gram of articular cartilage obtained from the metacarpophalangeal joints of yearling steers.

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### 3.3. Expansion Culture

With the current autologous chondrocyte implantation (ACI) approach, insufficient chondrocytes are obtained from primary isolation for direct implantation. Therefore, the primary chondrocyte population requires ex vivo expansion before use for tissue repair or engineering. Based on information derived from Brittberg et al. [1994] and from literature provided by Genzyme Tissue Repair, the current ACI protocol involves in vitro expansion of 180,000–455,000 primary cells over 2–5 weeks to provide sufficient cells for implantation ($2.6–5.0 \times 10^6$ chondrocytes

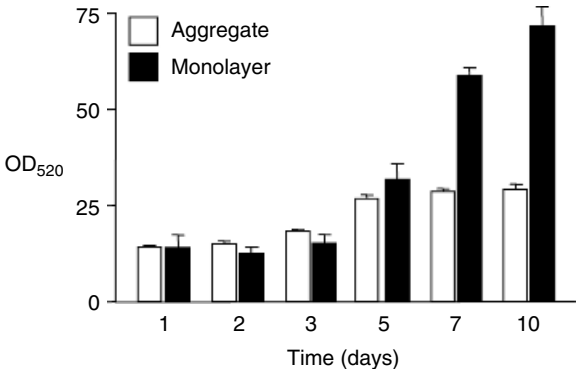


**Figure 4.1.** In vitro proliferation of articular chondrocytes. Bovine articular chondrocytes were cultured as aggregates (white bars) or monolayers (black bars) in expansion medium for up to 10 days. DNA content was measured by Pico Green fluorescence (Molecular Probes, Eugene, OR). Chondrocytes cultured as aggregates undergo a single population doubling in the first 5 days in culture and then cease proliferative activity. In contrast, monolayer cultures maintain proliferative activity throughout the culture period, with approximately sixfold increase in cell number by day 10 ($n = 4$).

in Brittberg et al., $1.2 \times 10^7$ chondrocytes in Genzyme's "Carticel" protocol). This requires 25–30 cell doublings.

Conditions that maintain the differentiated articular chondrocytic phenotype in vitro (See below) do not support sustained proliferation (Fig. 4.1). The standard technique for articular chondrocyte expansion involves seeding isolated chondrocytes as subconfluent monolayers in the presence of serum and/or specific mitogens. Articular chondrocyte monolayers become confluent at a density of $2.5–3.0 \times 10^5$ cells/cm$^2$. Therefore, for expansion, primary cells are usually seeded at an initial density of $1.0–2.5 \times 10^4$ cells/cm$^2$, allowing for 10- to 25-fold increases in cell number before confluence. Monolayer expansion results in loss of expression of genes that characterize the articular chondrocytic phenotype [Stewart et al., 2000]. The rate at which the differentiated phenotype is lost is dependent on seeding density.

Protocol 4.3 details establishment of primary chondrocyte monolayer cultures and subculture of chondrocyte monolayers. The particular culture vessels used (cell culture dishes, capped flasks, etc) will depend on the starting cell number, required expansion indices, biological safety requirements, and laboratory preference. The protocol assumes use of tissue culture flasks.

## Protocol 4.3. Monolayer Culture of Articular Chondrocytes

### *Reagents and Materials*

*Sterile*

- ❑ Ascorbic acid stock (5 mg/ml) (See Section 2.5)
- ❑ Differentiation medium (See Section 2.6)

- ❑ Growth medium (See Section 2.7)
- ❑ PBSA
- ❑ Trypsin-EDTA: trypsin, 0.25%, EDTA, 1 mM in PBSA
- ❑ Plastic centrifuge tube, 50 ml (e.g., Falcon)

***Protocol***

(a) Resuspend the chondrocytes in growth medium by gentle trituration. The volume of medium will depend on the starting cell number and the required cell concentration for the experiment. For example, if chondrocytes are to be seeded in 75 $cm^2$ flasks (20 ml/flask) at an initial density of $2.5 \times 10^4$ cells/$cm^2$, each flask will require $1.875 \times 10^6$ cells, suspended in 20 ml of culture medium. Periodic resuspension of the chondrocytes is recommended to keep the cell density consistent.

(b) Maintain cultures at 37 °C in a 95% air-5% $CO_2$ humidified incubator.

(c) Refeed the cells with fresh growth medium every 2–3 days, as required, until confluence. The time required to reach confluence varies considerably, depending on donor age. A minimum of 4 days would be expected for a fourfold increase in cell density.

(d) At confluence, aspirate the medium and rinse the cell layer with PBSA.

(e) Add approximately 1.0 ml trypsin/EDTA per 20 $cm^2$. Tip the flask to distribute the trypsin-EDTA across the entire surface of the cell layer and incubate at 37 °C for 5–10 min, or until the cells begin to detach.

(f) Dislodge the chondrocytes from the surface by lightly tapping the bottom of the flask against a hard surface.

(g) Add 10 volumes of growth medium and complete the cell dislodgement and resuspension by vigorous pipetting.

(h) Transfer the suspension to a Falcon tube. Rinse the flask with 3 ml medium and transfer the rinse to the Falcon tube.

(i) Pellet the cells by centrifugation at 250 *g* for 5 min.

(j) Resuspend the chondrocytes in fresh culture medium and count the cells with a hemocytometer.

(k) In some instances, chondrocyte monolayers detach as a single sheet and do not dissociate into single cells. Secondary digestion of the chondrocyte layer in 0.05% collagenase A for 1–2 h, similar to the protocol provided for primary chondrocyte isolation (Protocol 4.1), provides single-cell suspensions.

(l) Reseed the passaged chondrocytes into flasks at the appropriate density.

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In the expansion protocol detailed above, serum is used as the source of mitogenic stimuli for chondrocyte proliferation. A considerable amount of research has been carried out to identify specific growth and differentiation factors [Bradham and Horton, 1998; Pei et al., 2002] and culture conditions [Domm et al., 2002; Kuriwaka et al., 2003; Murphy and Sambanis, 2001] that can be used with, or in place of, serum to stimulate chondrocyte proliferation while mitigating effects

on the differentiated phenotype and/or facilitating restoration of the differentiated phenotype after expansion. In particular, fibroblast growth factor seems to have a specific phenotype-sparing activity during chondrocyte expansion [Jakob et al., 2001; Mandl et al., 2002; Martin et al., 1999]. It should also be noted that recent studies have demonstrated that the capacity of monolayer-expanded chondrocytes to regain the differentiated chondrocytic phenotype is lost as the number of passages increases [Dell'Accio et al., 2001; Schulze-Tanzil et al., 2002].

### 3.4. Differentiation/Redifferentiation Culture Models

Three-dimensional (3D) culture conditions are required for the maintenance or restoration of a differentiated articular chondrocytic phenotype. Low-serum or serum-free conditions are also preferable, but the effects of serum supplementation in 3D culture are considerably less than in monolayer culture [Stewart et al., 2000]. Reexpression of the differentiated phenotype generally requires 3D culture for 2–3 weeks. Several approaches have been developed for the 3D maintenance of chondrocytes (Fig. 4.2). Micromass [Bradham and Horton, 1998; Schulze-Tanzil et al., 2002], aggregate [Reginato et al., 1994; Stewart et al., 2000], and pellet [Ballock and Reddi, 1994] models are simple procedures that do not require addition of a substrate to the cells. The micromass model involves seeding small numbers of chondrocytes (approximately $2.0 \times 10^5$) in small volumes at superconfluent densities, inducing multicellular nodule formation. In practice, this technique generates a mixed population of 3D and monolayer cells and is not ideal for redifferentiation applications. The aggregate model involves culturing chondrocytes in nonadherent wells or dishes. These can be purchased (Corning-Costar ultra-low-attachment plates) or made by layering the bottom of regular dishes with methacrylate [Reginato et al., 1994; Stokes et al., 2002] or agarose [Archer et al., 1990; Kolettas et al., 1995]. The aggregate model is ideal for maintaining the differentiated phenotype of primary chondrocytes. It is less effective for restoring the differentiated phenotype of monolayer-expanded chondrocytes because the characteristic aggregation that occurs with primary cells is attenuated after monolayer expansion. For this application, the pellet model (Protocol 4.4) is more effective.

Alginate [Bonaventure et al., 1994; Gagne et al., 2000; Häuselmann et al., 1994; Homicz et al., 2003; Mandl et al., 2002; Masuda et al., 2003; Murphy and Sambanis, 2001], agarose [Benya and Shaffer, 1982; Lee et al., 1998; Weisser et al., 2001], fibrin [Fortier et al., 2002; Hendrickson et al., 1994], and collagen [Kuriwaka et al., 2003; Lee et al., 2003] models involve suspension of the chondrocytes within a 3D substrate or gel. The substrate-based models are particularly suitable for tissue engineering applications because phenotypic redifferentiation and generation of an engineered construct for implantation can occur simultaneously [Chang et al., 2001; Marijnissen et al., 2002; Masuda et al., 2003; Miralles et al., 2001; Weisser et al., 2001]. The alginate model is particularly versatile in that the chondrocytes can be reisolated from the construct by calcium chelation [Masuda et al., 2003] (Protocol 4.5).

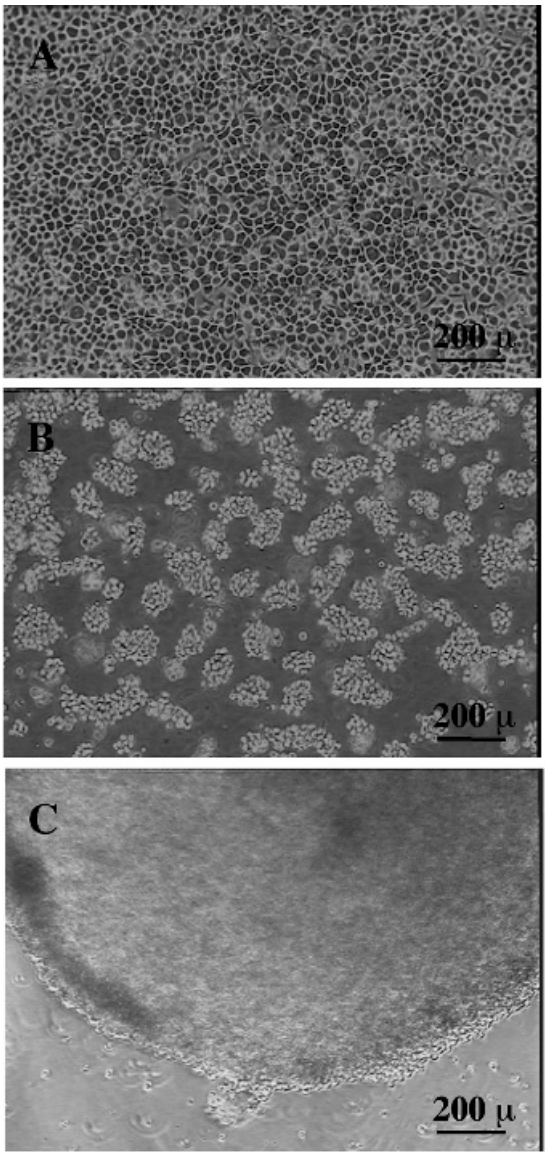


**Figure 4.2.** Morphological appearance of chondrocyte cultures. Images were obtained 48 hours after bovine articular chondrocyte cultures were established as high-density monolayers (A), aggregates (B), or pellets (C).

## Protocol 4.4. Pellet Culture for Chondrocytes

### *Reagents and Materials*

*Sterile*

- ❑ 15 ml conical polypropylene centrifuge tubes

- ❑ Ultra-low-attachment plates
- ❑ Differentiation medium (See Section 2.6)

***Protocol***

(a) Resuspend the chondrocytes in differentiation medium at 160,000–250,000 cells/ml.
(b) Dispense 1-ml aliquots of the resuspended cells into 15-ml conical polypropylene centrifuge tubes.
(c) Centrifuge the tubes at 250 *g* for 5 min at 4 °C.
(d) Loosen the tube caps to allow gas exchange and maintain at 37 °C in a 95% air-5% $CO_2$ humidified incubator.
(e) Refeed the pellets every 2–3 days by gently aspirating 80% of the medium. Particular care should be taken to avoid disturbing the pellet during refeeding.
(f) After 3–5 days, the cell pellets will have consolidated sufficiently to permit direct handling.

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Pellet cultures can also be established by adding $2.5 \times 10^5$ cells in 500 μl of medium to 1.5 ml microfuge tubes and then centrifuging the tubes in a Beckman 12 horizontal centrifuge at 250 *g* for 5 min. After centrifugation, the microfuge tube caps are pierced with an 18 g needle to allow gas exchange and the tubes are maintained in racks at 37 °C in a 95% air-5% $CO_2$ humidified incubator. The pellets can be maintained in the microfuge tubes for the duration of the experiment, or transferred to nonadherent plates after 3 days.

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## Protocol 4.5. Alginate Culture of Chondrocytes

***Reagents and Materials***

*Sterile*

- ❑ Alginate solution (See Section 2.8)
- ❑ Syringe, 5 or 10 ml
- ❑ Hypodermic needle, 22 g
- ❑ $CaCl_2$ solution, 102 mM (See Section 2.9)
- ❑ Growth medium (See Section 2.7) or differentiation medium (See Section 2.6)
- ❑ Saline, 0.9% (See Section 2.10)
- ❑ Alginate depolymerization solution (See Section 2.11)

***Protocol***

(a) Resuspend the cells in alginate solution. Cell concentrations ranging from $1 \times 10^3$ to $1 \times 10^7$ cells/ml have been reported in the scientific literature [Gagne et al., 2000], with $4 \times 10^6$ cells/ml being standard [Binette et al., 1998; Häuselmann et al., 1994; Masuda et al., 2003].
(b) Aspirate the suspension into a syringe.

(c) Dispense the cell suspension as drops through a 22 g hypodermic needle into 102 mM $CaCl_2$ solution.
(d) Allow the alginate beads to polymerize for 10 min.
(e) Rinse the beads twice with 0.9% sterile saline and then with culture.
(f) Immerse the beads in culture medium and maintain at 37 °C in a 95% air-5% $CO_2$ humidified incubator.
(g) Change culture medium every 2–3 days for the duration of the experiment.
(h) The chondrocytes and associated pericellular matrix can be released by dissolving the alginate in alginate depolymerization solution for 20 min.
(i) Transfer the solution to a centrifuge tube and spin at 200 *g* for 10 min at 4 °C to pellet the cells with associated pericellular matrices.

The assays routinely used to assess in vitro phenotypic modulation of chondrocytes and chondrocyte progenitors primarily assess cell morphology and the expression of genes, proteins, and proteoglycans representative of the cartilaginous matrix. The use of these assays for tissue engineering purposes assumes that the qualitative (re)appearance of specific marker expression indicates comprehensive restoration of the differentiated chondrocytic phenotype and that in vitro behavior reflects in vivo reparative or regenerative capacity. Recent studies suggest these assumptions may not be correct. First, "redifferentiation" does not restore expression profiles of primary chondrocytes [Benz et al., 2002; Domm et al., 2002]. Further, in vitro phenotypic modulation is not directly indicative of in vivo behavior [Dell'Accio et al., 2001]. Clearly, caution must be exercised in extrapolating selective in vitro data to general assumptions about "phenotype" and anticipated in vivo performance.

## 4. CELLS WITH CHONDROGENIC DIFFERENTIATION POTENTIAL

Many postnatal tissues contain cells that have chondrogenic differentiation potential, but they are not necessarily easily accessible for harvesting chondroprogenitors if autograft tissue engineering is the goal. Tissues such as bone marrow, fat, skin, and possibly synovium may be of practical use for the isolation of chondroprogenitors for cartilage tissue engineering. Cells isolated from each of these tissues have been shown to possess chondrogenic differentiation potential [Erickson et al., 2002; Johnstone et al., 1998; Nishimura et al., 1999; Yates et al., 2001]. The majority of the progenitor cell isolation procedures for tissue engineering used to date have been relatively straightforward because they have not involved steps to specifically purify the chondroprogenitor cells from other cell types. One reason for this has been the lack of specific markers for these cells. As with any new scientific field, the procedures are becoming more sophisticated, with enhanced purification schemes for progenitor cells based on various parameters becoming available. Use

of cell sorting, differential plating and/or altered medium conditions have produced enrichment of cells with differing differentiation potentials [Gronthos et al., 2003; Jiang et al., 2002; Jones et al., 2002; Reyes et al., 2001; Sekiya et al., 2002; Vacanti et al., 2001]. Although these procedures are now available, there are not yet any published studies demonstrating the benefit of the purification schemes to tissue-engineered implant production.

Most of the purification schemes have been worked out with bone marrow as the source of cells. The ease of harvest and relative ease of expansion of marrow-derived cells make them good candidates for tissue engineering of musculoskeletal tissues. Bone marrow can be accessed in most patients as an autologous cell source. The isolation of mesenchymal progenitor cells from bone marrow is described extensively in Chapter 2. It is known that the number of marrow-derived progenitor cells has been shown to decrease with age [Bergman et al., 1996; D'Ippolito et al., 1999; Nishida et al., 1999]. Although this has been reported to be a potential limitation of marrow as a source of these cells, it may also be true of all other tissues, too; it has been also noted in periosteum but not determined for other sources to date [O'Driscoll et al., 2001].

Periosteum contains osteochondral progenitor cells that can differentiate into osteoblasts and chondrocytes during normal bone growth and fracture healing. Detailed experimental knowledge of periosteal chondrogenesis has been gained from in vitro culture and chondrogenic induction of periosteal explants, in a system developed by O'Driscoll and others [Fukumoto et al., 2002; Miura et al., 1994, 2002; Mizuta et al., 2002; Mukherjee et al., 2001; O'Driscoll et al., 1994, 1997, 1998, 2001; Sanyal et al., 2002; Saris et al., 1999]. This system of culture has been used as an assay for the chondrogenic capacity of other tissues [Nishimura et al., 1999]. Isolated periosteal cells can be induced to undergo chondrogenesis [Izumi et al., 1992; Nakahara et al., 1991], and both periosteum and isolated periosteal cells with chondrogenic capacity have been used for articular cartilage and meniscus repair experiments [Kobayashi et al., 2002; Mason et al., 1999; O'Driscoll, 1999; O'Driscoll and Fitzsimmons, 2001; Perka et al., 2000; Rubak et al., 1982; Walsh et al., 1999]. Periosteal tissue is also used as a cover for articular cartilage defects into which chondrocytes are placed [Brittberg et al., 1994, 1996; Minas and Nehrer, 1997]. Periosteal cells are not an obvious choice as a source for cells for in vitro tissue engineering strategies unless exposure of bone is done in preliminary surgery.

Although termed synoviocytes, the population of cells isolated and expanded from synovial tissue contains several cell types. The cells of the synovial intima are thought to be a mixture of bone marrow-derived macrophages (type A cells) and specialized fibroblasts (type B cells) [Edwards, 1994]. In the subintimal layers there is connective tissue with blood vessels, nerves, and lymphatics. Although some researchers use approaches that attempt to isolate only the intima, it is difficult to do this precisely. Within the mixed population of cells termed synoviocytes are chondroprogenitor cells. Synovium-derived cells undergo chondrogenic differentiation when cultured on bone morphogenetic protein-coated plates [Iwata et al., 1993] and

in in vitro chondrogenesis assays of the type described in Protocol 4.1 [Nishimura et al., 1999]. Recently, these cells have been shown to also have osteogenic, adipogenic, and myogenic potential [De Bari et al., 2001, 2003]. The presence of progenitor cells in synovium fits with the clinically noted formation of multiple cartilaginous nodules in the synovium (synovial chondromatosis), a benign reactive metaplasia of synovial cells with unknown etiology [Crone and Watt, 1988]. Furthermore, rheumatoid pannus has been shown to contain chondrocyte-like cells, which might also arise from synovial progenitor cells [Allard et al., 1988; Xue et al., 1997]. Biopsy procedures may allow the use of autograft synovium for isolation and expansion of synovial progenitor cells for use in tissue engineering.

Both fat [Erickson et al., 2002; Tholpady et al., 2003; Winter et al., 2003; Young et al., 1993; Zuk et al., 2001, 2002] and dermis [Mizuno and Glowacki, 1996a,b; Yates et al., 2001; Young et al., 2001] have been demonstrated to contain chondroprogenitor cells. Both of these tissues could be practically harvested for isolation of autologous progenitor cells for tissue engineering. The chondrogenic extracellular matrix formed by cells from these sources has not been shown to be extensive. It is noted that dilution of bone marrow-derived mesenchymal progenitor cells with dermal fibroblasts decreases chondrogenesis [Lennon et al., 2000], so it may that the relatively simple isolation procedures used to date may include too many contaminating nonchondrogenic cells. Characterization of the progenitor cells present in these tissues [Gronthos et al., 2001] may allow better purification of the subpopulation with chondrogenic differentiation potential, which may then provide for extensive cartilage production.

## 5. CHONDROGENESIS OF PROGENITOR CELLS IN VITRO

Until the late 1990s, the chondrogenic assays for mammalian progenitor cells isolated from postnatal tissues consisted of various methods for implantation of the cells in vivo, generally in scaffolds or diffusion chambers [Brown et al., 1985; Cassiede et al., 1996; Goshima et al., 1991; Harada et al., 1988; Jaroma and Ritsila, 1988; Nakahara et al., 1990]. The implants were then harvested and submitted for histologic evaluation of the tissues formed. This implantation method has been used for "proof of concept" studies of chondrocytes in various scaffold types [Cao et al., 1997; Isogai et al., 1999; Paige et al., 1996; Sims et al., 1996]. However, although it can be used for assessing the differentiation potential of cells, it is limited for tissue engineering with chondroprogenitor cells. Thus the development of in vitro systems for inducing chondrogenesis was important.

A method based on the pellet culture system that was first used for the culture of differentiated chondrocytes was developed [Johnstone et al., 1998; Yoo et al., 1998]. Pellet culture was first described by Holtzer et al. [1960] for culturing chondrocytes in conditions that maintained their differentiated phenotype. It was later adapted for use with growth plate chondrocytes and allowed the study of the differentiation of proliferative chondrocytes into the hypertrophic state [Ballock et al., 1993; Chen et al., 1995; Inoue et al., 1990; Iwamoto et al., 1991; Kato et al., 1988].

The appeal of the system for use with progenitor cells was its use of two parameters long known to be important for chondrogenesis: high cell density and lack of cell-substratum interactions, such as would occur in monolayer conditions [Solursh, 1991]. Ballock et al. published a method for pellet culture of growth plate chondrocytes with a serum-free defined medium [Ballock et al., 1993; Ballock and Reddi, 1994]. This formed the basal medium in which successful chondrogenesis of progenitor cells was first achieved [Johnstone et al., 1998]. The addition to the medium of dexamethasone [Johnstone et al., 1998] and TGF-$\beta$1 [Johnstone et al., 1998; Yoo et al., 1998] facilitated the differentiation of chondroprogenitor cells within the adherent cell population isolated from bone marrow (Fig. 4.3).

The sequence of chondrogenesis induced in these conditions begins with the aggregation of the cells, forming a free-floating ball, generally within 12 hours of initiation (Fig. 4.4). There is a condensation of this cell mass within the first 2–3 days and then a gradual expansion in size as the cells undergo chondrogenic differentiation and produce extracellular matrix (Fig. 4.5, See Color Plate 3). At day 1, the aggregated cells vary greatly in morphology. Some of the cells are arranged in a syncytium, whereas others will be individually delineated. At the periphery of the aggregates a few of the cells are already elongated (fusiform). By day 5, there is an increase in fusiform cells throughout the aggregate, but especially at the periphery, where they form a multilayered zone. Matrix production increases especially in the middle of the aggregate. By day 7, the center of the aggregate consists of more rounded cells. By day 14, the cells of the center of the pellet stand out as individual entities surrounded by extracellular matrix, which exhibits metachromasia with Toluidine Blue staining.

Peripheral layers of flattened cells are noted in other types of culture that allow chondrogenic differentiation, such as the micromass cultures of chick and mouse cells used for studies of the mechanism of chondrogenesis [Ahrens et al., 1977; Cottrill et al., 1987; Gluhak et al., 1996; Merker et al., 1984]. Although these layers resemble a perichondrium in appearance, there is no evidence for any activity that would be associated with a perichondrial layer. It is noted that the formation of the

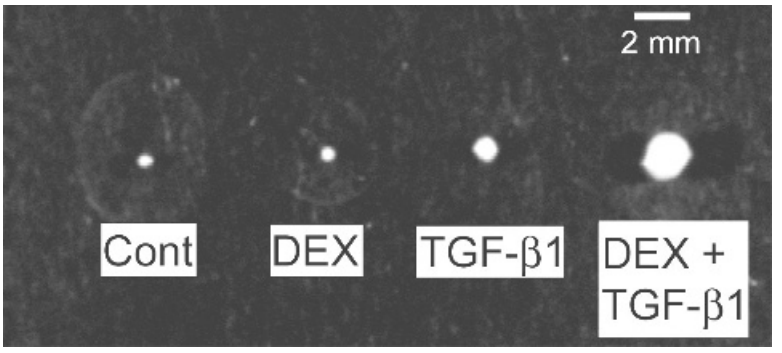


**Figure 4.3.** Aggregates of bone marrow-derived cells. Culture after 14 days in defined medium (Cont) without dexamethasone or with additions as labeled (Dex: dexamethasone).

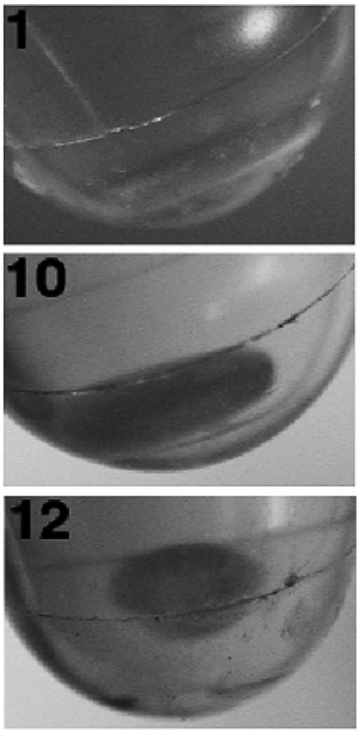


**Figure 4.4.** Sequence of aggregation. The sequence of aggregation from 1 hour to 12 hours after centrifugation of human marrow-derived cells in chondrogenic medium.

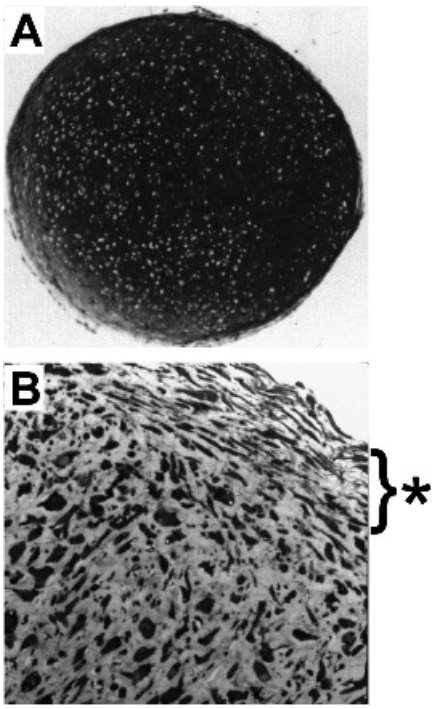


**Figure 4.5.** The Toluidine Blue metachromatic matrix of cartilaginous aggregates after 14 days in chondrogenic medium. A) Section of paraffin-embedded whole aggregate of human marrow-derived cells. B) Higher-magnification image of edge of a methyl methacrylate embedded section with the region of flattened cells indicated (*). The preservation in the methyl methacrylate embedding process allows a better appreciation of the different regions and the extent of cartilage extracellular matrix production. (See Color Plate 3.)

flattened peripheral layers is also seen when clonal chondroprogenitor cell lines are cultured in the same system [Dennis et al., 1999], arguing against the idea that cell sorting is the basis for the development of these layers of cells that do not undergo differentiation.

## Protocol 4.6. In Vitro Chondrogenesis

***Reagents and Materials***

*Sterile*

- ❑ Trypsin-EDTA: 0.25% trypsin, 1 mM EDTA in PBSA
- ❑ L-Ascorbate-2-phosphate stock (See Section 2.12)
- ❑ Dexamethasone ($10^{-3}$ M) stock (See Section 2.13)
- ❑ Transforming growth factor $\beta$-1 (TGF-$\beta$1) stock (See Section 2.14)
- ❑ Basal medium (See Section 2.15)
- ❑ 15-ml conical polypropylene centrifuge tubes

***Protocol***

(a) Trypsinize cells from monolayer and count.
(b) Calculate the number of pellets to be made (typically $2.0 \times 10^5$ cells per pellet, $4.0 \times 10^5$ cells/ml) and make up the appropriate amount of differentiation medium by adding L-ascorbate-2-phosphate stock, dexamethasone stock, and TGF-$\beta$1 stock in a 1:100 ratio to basal medium.
(c) Aspirate trypsin solution and resuspend cells at $4.0 \times 10^5$ cells/ml.
(d) Dispense 0.5-ml aliquots of the resuspended cells into 15 ml conical polypropylene centrifuge tubes.
(e) Centrifuge the tubes at 500 *g* for 5 min at 4 °C.
(f) Loosen the tube caps to allow gas exchange and maintain at 37 °C in a 95% air-5% $CO_2$ humidified incubator.
(g) Refeed the pellets every 2–3 days by gently aspirating 80% of the medium. Particular care should be taken to avoid disturbing the pellet during feeding.
(h) After 2 days, the cell pellets will have consolidated sufficiently to permit direct handling.

Since the introduction of this in vitro assay, several variations have been developed. Although done for improvement of the in vitro assay as performed by a given laboratory, these alterations may be exploited for tissue engineering if they result in greater extracellular matrix production or a more appropriate matrix constitution for the desired tissue. Barry et al. [2001] noted that use of TGF-$\beta$3 provided them with greater matrix elaboration. Sekiya et al. [2001] produced extensive chondrogenesis when BMP-6 was also added. Although dexamethasone is needed for

initiation of chondrogenesis in this system, withdrawal of dexamethasone from the culture after 7 days promotes greater extracellular matrix production [Roh et al., 2001].

Regardless of the exact composition of the inductive medium, the cartilage produced in this manner is probably best described as a fibrocartilage, because there is type I collagen produced at all times during the culture. Clearly, the center of a pellet is more hyaline and the outer layers are more fibrous (Fig. 4.5, See Color Plate 3), but this method does not produce a cartilage that is identical to any of the cartilages found in the body, which have individual features based on their extracellular composition and organization. One other feature of the system that should also be noted is the progression of the cells through chondrogenesis to become hypertrophic chondrocytes, expressing type X collagen, that will die by apoptosis [Johnstone et al., 2002]. Although a good feature of the system for exploring differentiation, the production of hypertrophic cartilage is not necessarily desired, depending on the type of cartilage that is the repair goal. This aspect is discussed further in Section 6.

## 6. TISSUE ENGINEERING CARTILAGE IMPLANTS FROM CELLS WITH CHONDROGENIC POTENTIAL

Although cartilaginous aggregates of up to $5.0 \times 10^5$ cells can be produced with this in vitro assay system, these are very small (Fig. 4.3) and not of obvious use for tissue engineering cartilage as is. Modification of the in vitro assay system of progenitor cells to produce tissue-engineered cartilage for implantation has been the subject of many recent studies [Angele et al., 1999, 2000; Martin et al., 2001; Noth et al., 2002], and the subject is covered in depth in Chapters 6 and 8. All the considerations associated with tissue engineering of any tissue are relevant: use of scaffolds, bioreactors, mechanical conditioning of the implant, etc. It is not clear what stage of cell differentiation, matrix production, and matrix organization will be most appropriate in implants for each type of cartilage. Furthermore, we do not yet know exactly how to achieve some of the desired types of cartilage. For example, one of the challenges for production of articular cartilage implants with progenitor cells is the facilitation of chondrogenic differentiation without hypertrophy. Although many of the factors that are involved in controlling chondrocyte differentiation are known, the appropriate stimuli that produce articular chondrocytes are still being defined. For fibrocartilage implants, such as those for meniscus, controlling the production of large amounts of collagen fibers in appropriate ratio to proteoglycans and other matrix molecules is still a challenge. Cartilage tissue engineering is a relatively new field, and it needs to become more sophisticated. It is expected that substantial progress will be made in this area in the next few years.

## SOURCES OF MATERIALS

| *Material* | *Catalog #* | *Supplier* |
|---|---|---|
| Alginate (Keltone LV) | N/A | ISP |
| L-Ascorbic acid-2-phosphate | AC358610250 | Fisher Scientific |
| Bovine serum albumin | A 9647 | Sigma |
| $CaCl_2$ | C69-500 | Fisher Scientific |
| Cell culture flasks, 75 $cm^2$ | 430641 | Corning |
| Cell culture flasks, 25 $cm^2$ | 430639 | Corning |
| Petri dishes, 10 cm | 3160-100 | Corning |
| Centrifuge tubes, 15 ml conical polypropylene | 352095 | Becton Dickinson |
| Centrifuge tubes, 50 ml conical polypropylene | 352079 | Becton Dickinson |
| Collagenase A | 1088785 | Roche Diagnostics |
| Collagenase type II | LS004176 | Worthington Biochemical Corp |
| Cryotubes | 3471 | CLP |
| DMEM, 4.5 g/l glucose, containing pyruvate and glutamate | 10569-010 | GIBCO Invitrogen |
| DMEM, 1.0 g/l glucose, containing pyruvate and glutamate | 11885-084 | GIBCO Invitrogen |
| Dexamethasone | D-4902 | Sigma |
| Fetal bovine serum | 16000-044 | GIBCO Invitrogen |
| Filter, 500 ml, 0.22 μm | SCGP UO5 RE | Millipore |
| Filter, 250 ml, 0.22 μm | SCGP UO2 RE | Millipore |
| Filter (Steriflip) 50 ml, 0.22 μm | SCGP 005 25 | Millipore |
| Filters, 40-μm tube | 352340 | Becton Dickinson |
| Fungizone™ antimycotic liquid | 15290-018 | GIBCO Invitrogen |
| Glutamine | 25-05-C1 | Cellgro |
| HCl | A1445 | Fisher |
| Ham's F-12 nutrient medium | 31765-035 | GIBCO Invitrogen |
| Hanks' balanced salt solution | 24020-117 | GIBCO Invitrogen |
| ITS-G Supplement (100×) | 41400-045 | GIBCO Invitrogen |
| ITS + Premix | 40352 | B-D Biosciences |
| Insulin-transferrin-sodium selenite supplement | 1074547 | Roche Diagnostics |
| Opti-MEM 1 reduced-serum medium | 31985-070 | GIBCO Invitrogen |
| Penicillin-streptomycin liquid | 15140-122 | GIBCO Invitrogen |
| PBS, $Mg^{2+}$, $Ca^{2+}$-free | 14190-144 | GIBCO Invitrogen |
| Pronase | 0165921 | Roche Diagnostics |
| NaCl | S671-500 | Fisher Scientific |
| Sodium pyruvate | 11360-070 | GIBCO Invitrogen |
| $C_6H_5O_7Na_32H_2O$ | S279-500 | Fisher Scientific |
| TGF-$\beta$1 | 240-B | R&D Systems |
| Trypan Blue | 15250-061 | GIBCO Invitrogen |
| Trypsin-EDTA: 0.25% trypsin, 1 mM EDTA | 25200-056 | GIBCO Invitrogen |
| Ultra-low-attachment plates, 35 mm | 3471 | Costar |

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