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Isolation of adipose derived stem cells and their induction to a chondrogenic phenotype

Bradley T. Estes[†], **Brian O. Diekman**[†], **Jeffrey M. Gimble**[**], and **Farshid Guilak**[†,*]

[†]Departments of Surgery and Biomedical Engineering, Duke University Medical Center, Durham, North Carolina 27710 USA

[**]Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA 70808

Summary

The ability to isolate, expand, and differentiate adult stem cells into a chondrogenic lineage is an important step in the development of tissue engineering approaches for cartilage repair or regeneration for the treatment of joint injury or osteoarthritis, or for application in plastic or reconstructive surgery. Adipose-derived stem cells (ASCs) provide an abundant and easily accessible source of adult stem cells for use in such regenerative approaches. This protocol describes the isolation of ASCs from liposuction aspirate, as well as cell culture conditions for growth factor based induction of ASCs into chondrocyte-like cells. These methods are similar to those used for bone marrow mesenchymal stem cells but distinct due to the unique properties of ASCs. Investigators can expect consistent ASC differentiation, allowing for slight variation due to donor and serum lot effects. Approximately 10–12 weeks are needed for ASC isolation and the characterization of chondrocyte-like cells, which is also described.

INTRODUCTION

The treatment of pathologies in articular and elastic cartilage pose important unmet challenges to the medical community. For example, arthritis represents the most common cause of disability in the US, leading to joint pain and dysfunction in over 40 million Americans [1, 2] Osteoarthritis, the most common form of arthritis, involves degeneration of the articular cartilage, the smooth, load-bearing tissue lining the ends of long bones within the synovial joints of the body. The greatest risk factors for osteoarthritis include aging, obesity, joint trauma, and mutations in cartilage specific matrix proteins [3]. Current estimates on the treatment costs, both indirect and direct, of osteoarthritis in the US are escalating to greater than $65 billion annually [4]. For plastic and reconstructive surgery in the head and neck area, elastic cartilage is often needed for nose, ear, and trachea reconstruction [5]. Estimates in the number of procedures involving bone and cartilage replacement exceed one million procedures per year [6].

[*]Corresponding Author: Farshid Guilak, Orthopaedic Research Laboratories, Department of Surgery, Division of Orthopaedic Surgery, 375 MSRB, Box 3093, Duke University Medical Center Durham, North Carolina 27710, Phone: (919) 684-2521, Fax: (919) 681-8490, guilak@duke.edu.

AUTHOR CONTRIBUTIONS

BTE, BOD, JMG, and FG were involved in the development, testing, and troubleshooting of these protocols, as well as the writing of this manuscript. All authors contributed extensively to this work.

Competing interests statement:

The authors (BTE, JMG, and FG) have filed patent applications on some of the material contained in this article.

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Cartilage Properties and Current Treatment Options

The unique function and properties of cartilage are provided by the tissue's extracellular matrix, which is maintained by a population of cells known as chondrocytes. Due to the small volume of chondrocytes (2–5% by volume), as well as the avascular and aneural properties of the tissue, cartilage exhibits little to no intrinsic repair capabilities in response to injury or disease. Traditional efforts to treat cartilage damage include joint lavage, tissue debridement, microfracture of the subchondral bone, abrasion arthroplasty, or the transplantation of autologous or allogeneic osteochondral grafts [7–17]. While these procedures have yielded promising clinical results, they are generally not applicable for large cartilage defects or for degenerative joint diseases such as osteoarthritis [18–20]. One tissue engineering approach, autologous chondrocyte transplantation, has shown promising results in early clinical reports, [21, 22] but recent randomized, controlled trials suggest little difference in the efficacy of this procedure over microfracture of the subchondral bone [23]. In this regard, there has been significant interest in the development of new tissue engineering strategies for the repair or replacement of damaged or diseased cartilage, and integral to such approaches is the need for an abundant and easily accessible source of cells. While most early attempts at cartilage tissue engineering have relied on the use of primary differentiated chondrocytes, it is important to note that there are many issues associated with the harvest of autologous tissue and cells for repair of cartilage. Among these are the disease state of the harvested cells (from the inflamed joint), the potential for initiation of osteoarthritic changes in the joint [23, 24], the lack of adequate autogenous tissue, and difficulty in expanding the cells *ex vivo* while maintaining the chondrocyte phenotype [25, 26]. These issues provide significant barriers for the use of autogenous primary chondrocytes for successful cartilage repair treatment strategies. Currently, defects in the head and neck area are typically treated with autologous cartilage, usually harvested from costal cartilage grafts [27]. Advantageously, the use of autologous tissues presents an implant that can be formed in the correct anatomical shape without risk of immunological rejection. However, the limitations and potential disadvantages of autologous tissue use are noteworthy and include lack of available tissue for large defects, the risk of iatrogenic deformity, and significant donor-site morbidity [27, 28].

Stem Cells in Cartilage Repair

The use of adult stem cells presents a viable alternative for cartilage repair strategies and has the potential for success while avoiding the issues associated with autogenous grafts andor cells. Mesenchymal stem cells (MSCs) from bone marrow have been shown to possesses multilineage differentiation potential and have been characterized extensively for a variety of tissue engineering applications including chondrogenesis [29–31]. Another multipotent adult stem cell population, adipose derived stem cells (ASCs), can easily be obtained from liposuction waste [32, 33] and has been shown in numerous studies to exhibit the potential for chondrogenesis, osteogenesis, adipogenesis, myogenesis, and some aspects of neurogenesis [34–45]. While these cells show some similarities to bone marrow MSCs, they appear to have a number of distinct characteristics with respect to their cell surface markers, differentiation potential, and abundance in the body. For example, compared to 100 ml of bone marrow aspirate, up to 300-fold more stem cells can be obtained from 100 g of adipose tissue [31, 46].

ASCs have demonstrated significant potential for chondrogenic differentiation when expanded in appropriate monolayer conditions [47] and cultured in growth factor containing medium in 3D culture [34–36, 43, 45]. As it was originally hypothesized that ASCs were another source of MSCs, similar conditions used to induce chondrogenesis in MSCs [31] were employed for the ASC population [36, 43]. In optimizing ASC differentiation protocols, it was later discovered that other members of the transforming growth factor beta (TGF-β)

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superfamily, such as bone morphogenetic protein 6 (BMP-6), can serve as potent regulators of ASC chondrogenesis [45, 48]. In general, chondrogenesis requires a three-dimensional culture system, and ASCs can be successfully induced down a chondrogenic lineage in a variety of scaffold environments. These culture configurations include pellet culture, which takes advantage of cell-cell interactions in a similar fashion to condensation during cartilage development [49], alginate bead culture which employs an inert hydrogel to facilitate a rounded cell phenotype advantageous for chondrogenesis [36], and cartilage-derived matrix which seeks to recapitulate some of the cell-matrix interactions seen in native cartilage [50, 51].

To repair or regenerate articular cartilage, an understanding of the molecular constituents and their role in the mechanical function of cartilage must be taken into consideration. Articular cartilage provides joint congruity and a lubricated surface for articulation, effectively distributing loads of up to ten times body weight that pass through the joint during normal physiologic activity [52]. Remarkably, this tissue provides a nearly frictionless bearing surface for the joint and functions over decades of use with little or no wear under normal circumstances. Articular cartilage is > 60% collagen by dry weight [52, 53], primarily consisting of collagen type II with lesser amounts of other collagens (e.g., Types VI, IX, X, and XI). The collagen fibrils are located throughout the matrix and are intertwined with a highly concentrated negatively charged proteoglycan matrix [54, 55]. Two primary glycosaminoglycans (GAG) are found in articular cartilage, chondroitin sulfate and keratin sulfate. GAG side chains (polymer repeats of the sulfated molecule) are found assembled covalently to a protein core to form a proteolgycan aggregate (reviewed in [54]). The large aggregating proteoglycan, aggrecan, is assembled into a complex structure by a noncovalent linkage of these proteoglycan aggregates to a hyaluronate backbone, producing immobilized structures contributing to the articular cartilage solid matrix [53]. The assembly of the cartilage matrix gives the tissue its unique set of biomechanical properties by virtue of the ability of the collagen-proteoglycan matrix together with the interstitial fluid to effectively resist high levels of stress and strain engendered by normal loading of the joint. While creating a true mimic of articular cartilage may not be necessary for proper function, a tissue possessing similar molecular constituents will most likely possess similar mechanical properties. Thus, monitoring the assembly and spatial organization of key extracellular matrix components is an important step in the development of tissue-engineered cartilage implants.

Markers of Chondrogenesis

Herein, we report the methods for ASC isolation and expansion, scaffold preparation, encapsulation of cells, and biochemical conditions to induce chondrogenesis. See Figure 1 for a flow chart showing the procedure and associated timing information. We further report methods used to evaluate the degree of chondrogenic differentiation in 3D culture (Table 1).

Controls

We have previously shown that only 43% of ASCs at a clonal level are capable of chondrogenic differentiation [37]. Because of this, it is relatively straightforward to select cells that have both high and low chondrogenic differentiation potential, which can be used for positive and negative controls respectively. Also, in most circumstances, ASCs can be cultured in a control medium without growth factors to serve as a negative control [45, 51, 56, 57]. Specifically, as relates to histology and immunohistochemistry, sections of articular cartilage should be used as positive controls to assess the degree of chondrogenic differentiation of the tissue engineered constructs.

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Appropriate Selection of an ASC Culture System

In this protocol, we describe two commonly used 3D culture systems that can be employed to chondrogenically induce ASCs. While both of these culture systems have been successfully used for ASC chondrogenesis, the selection of one over the other depends highly on the study purposes. The use of pellet culture results in high spatial cell density and necessitates cell-cell contact, much like the cellular condensation process during limb development [49]. The use of alginate results in a rounded cell morphology, much like that observed in articular cartilage, which has also been shown to be an important factor in promoting ASC chondrogenesis [36]. The use of pellet culture mimics the development of cartilage during limb formation and is therefore often used as a method to understand the interaction of cells and growth and environmental factors to promote a chondrogenic phenotype [37, 48, 58, 59]. While alginate can also be used for this purpose [35, 36, 51, 56, 60], the use of alginate and other hydrogels can have profound influences on the ensuing phenotype of the cells, [35, 50, 61] and therefore, the effect of the biomaterial as a significant variable influencing the differentiation potential of the cells must be taken into account when choosing an appropriate culture system. It is also important to note that while we report on the methods for two often used cell culture systems, many other materials and culture systems have been reported for being supportive of ASC chondrogenesis [35, 50, 62] and should also be considered when selecting an ASC culture system. Regardless of the culture system employed, the methods for inducing chondrogenesis with the growth factors listed in Table 3 may still serve as a common starting ground for ASC chondrogenesis.

MATERIALS

REAGENTS

Cell isolation and expansion

- Adipose tissue (contains blood)

  Caution. See note below step 4 for guidelines in dealing with human tissue.

- 5, 10, and 25 ml serological Pipettes, sterile (Corning, cat. Nos. 4487, 4488, and 4489 respectively)
- 250 ml plastic bottles for centrifuging, sterile (Corning, cat. No. CLS430236)
- 50 mL polypropylene centrifuge tubes, sterile, (Corning, cat. No. 430290)
- 2 1L beakers, sterile
- 10% (v/v) bleach

  Caution. Corrosive. Causes eye, skin, and digestive tract burns. Harmful if inhaled and results in respiratory tract irritation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- 70% (v/v) ethanol

  Caution Ethanol is highly flammable. May also cause eye and respiratory tract irritation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- 10% (v/v) dish washing detergent
- Dulbecco's phosphate-buffered saline (D-PBS), without calcium chloride and magnesium chloride (Gibco, cat. no. 14190-144)
- BSA, Fraction V, 7.5% solution (Gibco, cat. No. 15260-037)

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- Type I Collagenase (Worthington Biochemical Products, cat. No. LS004197)

  Critical: We advise testing several lots of collagenase to insure effective digestion of the adipose tissue. There is significant variability between lots provided by a single commercial vendor.

- 1 M calcium chloride solution (sterile)
- 250 ml filter 0.22 μm low protein binding sterilization unit (Corning, cat. no. 430767)
- 225cm^2 Cell Culture Flask (Corning, cat. no. 431082)
- Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Broth (1:1. v/v) with 15 mM HEPES buffer, L-glutamine and pyridoxine hydrochloride (Gibco, Cat. No. 11330-032 or Biowhittaker, Cat No. 12-719F)
- Ammonium chloride (NH_4Cl) (Sigma, cat. No. A0171)
- Potassium carbonate (K_2CO_3) (Sigma, cat. No. P5833)

  Caution. Causes eye, skin, and respiratory tract irritation. Harmful if swallowed. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- Fetal Bovine Serum (Atlas Biologicals, Cat. No. F-0500-A)

  Critical: We advise testing several lots of serum for culture optimization, and then use of the same lot of FBS for an entire series of experiments. The authors have observed significant variability in the results obtained with different sera.

- 0.05% Trypsin/EDTA (Gibco, Cat. No. 15140-122)
- Penicillin/Streptomycin/Fungizone (Gibco, Cat. No. 15240-062)
- Human epidermal growth factor (rhEGF) (Roche, Cat. No. 1376454)
- Human fibroblastic growth factor, basic (rh-bFGF) (Roche, Cat. No. 1123149)
- Transforming Growth Factor beta-1 TGF-β1 (R&D Systems, Cat. No. 100-B-001)
- Cryogenic Vials (Corning, cat. No. 430488)
- Dimethylsulfoxide (DMSO) Hybri-Max® (Sigma, Cat. No. D2650)

  Caution Harmful if swallowed, inhaled, or absorbed through skin. Causes eye, skin, and respiratory tract irritation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- 1°C Freezing Container (Nalgene, Cat. No. 5100-0001))

Alginate bead culture

- Alginate (Pronova™ LVG USP, Cat. No. 4200001)
- Sodium Chloride (NaCl) (Sigma, Cat. No. S9625)
- Sodium Citrate Trisodium salt dihydrate (Sigma, Cat. No. S4641)
- Calcium Chloride (Sigma, Cat. No. C3306)
- Sterile Syringe filter (0.22 mm) (VWR International, Cat. No. 28145-477)
- 150 ml 0.22 mm filter system Corning, Cat. No. 431154).

- 24 well plate, with lid, flat bottom, Ultra-low attachment surface (Corning, Cat. No. 29443-032)
- VWR Spinbar® Micro stir bars (12.7 mm × 3 mm) (VWR International, Cat. No. 58948-397)

Chondrogenic Induction

- Dulbecco's Modified Eagles Medium-high glucose, (DMEM-HG), (Gibco, Cat. No. 11995-065)
- ITS+ supplement, (Collaborative Research, Cat. No. 40352)
- Dexamethasone, (Sigma, Cat. No. D-4032)
- L-Ascorbic acid 2-phosphate Sesquimagnesium Salt (Sigma, Cat. No. A8960)
- Penicillin/Streptomycin (Gibco, cat. No. 15140-122)
- Transforming Growth Factor beta-3 (TGF-β3), (R&D Systems, Cat. No. 243-B3-002)
- Bone Morphogenetic Protein-6 (BMP-6), (R&D Systems, Cat. No. 507-BP-020)
- Siliconized 200 μl Pipette tips (VWR, Cat. No. 53503-792)
- Siliconized 0.6 mL Snap-Cap microtubes (Sigma, Cat. No. T4691-500EA)
- 15 mL polypropylene centrifuge tubes, sterile, (Corning, cat. No. 430052)
- 50 mL polypropylene centrifuge tubes, sterile, (Corning, cat. No. 430290)

Papain Digestion solution

- L-Cysteine Hydrochloride Anhydrous (Sigma, Cat. No. C1276)

  Caution. Avoid contact. Causes eye, skin, and respiratory tract irritation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- Ethylenediaminetetraacetic acid, EDTA (Sigma, Cat. No. EDS-100G)

  Caution. Avoid contact. Causes eye, skin, and respiratory tract irritation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- Papain, 0.5 U/mg (Sigma, Cat. No. 76222)

  Caution. Avoid contact. Causes eye, skin, and respiratory tract irritation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- Sodium phosphate monobasic (Mallinckrodt, Cat. No. 7892)

  Caution. Avoid contact. Causes eye, skin, and respiratory tract irritation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

dsDNA Quantitation

- PicoGreen dsDNA Quantitation kit (Invitrogen, Cat. No. P-7589)

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DMB Assay

- Sodium Formate (Sigma, Cat. no. S2140)

  Caution. Harmful if swallowed or inhaled. Causes eye, skin, and respiratory tract irritation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- 1,9-Dimethyl-Methylene Blue (Sigma, Cat # 341088, Sigma)

  Caution. Known eye irritant. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves and safety glasses and allow for adequate ventilation.)

- Chondroitin 4-sulfate (C-4-S, type A - from Bovine Trachea) (Calbiochem, Cat. No. 230687)
- Formic Acid (EM Science, Cat. No. FX 0440-7)

  Caution. Harmful if swallowed or inhaled. Causes eye, skin, and respiratory tract irritation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- Clear, flat-bottom 96-well plate (BD, Cat. No. 353228)

Hydroxyproline assay

- Chloramine-T, hydrate 98% (Sigma, Cat. No. 857319)

  Caution. Harmful if inhaled. Substance is known to be destructive to the tissue of the mucous membranes and upper respiratory tract. Also causes eye and skin burns and may be harmful if swallowed. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- n-Propyl alcohol 1 L (Sigma, Cat. No. P6334)

  Caution. n-Propyl alcohol is highly flammable. Harmful if swallowed or inhaled or absorbed through skin. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- p-Dimethylaminobenzaldehyde (Sigma, Cat. No. D8904)
- Perchloric Acid 60% 1 lb (Fisher Scientific, Cat. No. A228)

  Caution. Harmful if swallowed or inhaled. Strong oxidizer. Corrosive. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood that is not designed for other uses.)

- Citric Acid Monohydrate (Sigma, Cat. No. C1909)

  Caution. Known eye irritant; results in severe eye irritation and possible injury. Also causes skin and respiratory tract irritation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- Sodium acetate Trihydrate (Sigma, Cat. No. S9513)
- Sodium Hydroxide Pellets (Mallinckrodt, Cat. No. 7708)

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Caution. Highly corrosive. Can result in eye and skin burns. May also result in respiratory and/or digestive tract irritation with possible burns. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- Acetic Acid Glacial (EM Science, Cat. No. UN 2789)

  Caution. Highly corrosive and flammable. Can result in severe burns to all body tissue and may be fatal if swallowed. Inhalation may cause lung and tooth damage. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- Hydrochloric Acid, 37% (6.0 N) (EM Sc*ience, Cat. No. UN1789*)

  Caution. Highly corrosive. Can result in severe burns to all body tissue and may be fatal if swallowed or inhaled. Inhalation may cause lung damage. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- trans-4 hydroxyproline-L (Sigma, Cat. No. H54409)
- Activated Charcoal Powder (EM Science, Cat. No. CX0645-1)
- 1.5 ml microcentrifuge tubes (VWR, Cat. No. 20170-038)
- Costar Spin-X HPLC micro centrifuge tube (nonsterile) and filter (0.45 μm nylon filter) (Corning, Cat. No. 8170)

Fixation

- 16% paraformaldehyde (Electron Microscopy Sciences, Cat. No. 15710)

  Caution. Paraformaldehyde is a suspected carcinogen. Avoid contact and inhalation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- Sodium Cacodylate, Trihydrate (Electron Microscopy Sciences, Cat. No. 12300)

  Caution. May be fatal if swallowed or inhaled. Harmful if absorbed through skin. Contains arsenic which can cause cancer. Skin, eye, and respiratory tract irritant. (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- Barium chloride (Mallinckrodt, Cat. No. 3756)

  Caution. Known irritant. May cause eye, skin, and respiratory tract irritation. May be fatal if swallowed or inhaled. Avoid contact and inhalation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

Histology

- Xylene (Mallinckrodt, Cat. No. 8668-16)

  Caution. Harmful or fatal if swallowed. Causes severe eye irritation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- Paraplast® Tissue Embedding Medium (Fisher Scientific, Cat. No. 23-021-399)

  Caution Paraffin burns readily. Keep away from fire.

- Tissue-Tek® Uni-Cassette® LWS (Sakura, Cat. No. 4156-02)

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- Fisherbrand® Superfrost®/Plus Microscope Slides (Fisher Scientific, Cat. No. 12-550-15)
- Safranin-O (Sigma, Cat. No. HT90432-1L)

  Caution. Causes eye, skin, and respiratory tract irritation. Avoid contact and inhalation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask).
- Toluidine blue (Sigma-Aldrich, cat. no. 89640-5G)

  Caution May cause gastrointestinal and blood disorders. Avoid contact and inhalation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)
- Fast Green FCF (Sigma, Cat. No. XXXXF7252-5G)

  Caution May be harmful if swallowed or inhaled. May cause eye, skin, and respiratory tract irritation. Avoid contact and inhalation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)
- Weigert Hematoxylin Solution (Sigma, Cat. No. HT 1079)

  Caution. Known irritant. May cause eye, skin, and respiratory tract irritation. Avoid contact and inhalation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)
- Differentiation solution (Sigma, Cat. No. A3179)

  Caution. Corrosive. Flammable; keep away from fire. Avoid prolonged exposure. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)
- Permount® (Fisher Scientific, Cat. No. SP15-100)

  Caution. Contains toluene. Causes eye, skin, and respiratory tract irritation. Avoid contact and inhalation. Inhalation may cause drowsiness and dizziness. May cause central nervous system depression. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

Immunohistochemistry

- Zymed Histostain® Plus Broad Spectrum (Invitrogen, Cat. No. 85-8943)
- Type II collagen antibody (Developmental Studies Hybridoma Bank, Cat. No. II-II6B3 ordered as supernatant)
- Type I collagen antibody (AbCam, Cat. No. AB6308)
- Type X collagen antibody (Sigma, Cat. No. C7974)
- 2-B-6 Chondroitin-4-Sulfate antibody (Seikagaku, Cat. No. 270432)
- 3-B-3 Chondroitin-6-Sulfate antibody (Seikagaku, Cat. No. 270433)
- Anti-Mouse IgG (Fab specific)–Biotin secondary antibody produced in goat (Sigma, Cat. No. B7151)
- Xylene

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Caution. Harmful or fatal if swallowed. Causes severe eye irritation. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)

- AEC Substrate-Chromagen (Invitrogen, Cat. No. 00-1111)
- Digest-All™ 3 (Pepsin) (Invitrogen, Cat. No. 00-3009)
- Chondroitinase ABC (Sigma, Cat. No. C 2905)
- PAP Pen (Research Products International, Cat. No. 195505)
- Methanol (EMD, Cat. No. MX0485-7)

  Caution. Harmful if swallowed. Highly flammable; keep away from heat and ignition sources. Use appropriate personal protective equipment to avoid exposure (i.e., wear gloves, safety glasses and a mask, or use material in fume hood.)
- Hydrogen Peroxide (EMD, Cat. No. HX0635-2)
- Goat serum (Invitrogen, Cat. No. 50-062Z)
- GVA Mounting Medium (Invitrogen, Cat. No. 008000)

qPCR

- RNASecure™ Reagent (Applied Biosystems, Cat. No. AM7005)
- 2 mL microcentrifuge tubes (RNase/DNase free) (VWR, Cat. No. 87003-298) Note: the design of these microcentrifuge tubes (wider bottom) allows more efficient chelation of alginate.
- Table 2: TaqMan Assays on Demand for assessing chondrogenesis [45]

Equipment

- Centrifuge
- Water bath shaker
- Microplate reader (for fluorescence and absorbance based assays)
- Speedvac or lyophilizer
- Microtome
- Real-Time PCR Instrument
- Hot plate with temperature control

REAGENT SETUP

Cell Isolation—Expansion medium: Use all reagents listed in Table 1. Expansion medium may be stored at 4° C in the dark for up to 2 weeks.

Stromal medium: Use all reagents listed in Table 1, except for rhEGF, rhFGF, and TGF-β1 (i.e., stromal medium should not contain any growth factors). Stromal medium may be stored at 4° C in the dark for up to 6 weeks.

Collagenase solution: This should be made fresh before starting the isolation and filter sterilized using a 250 ml sterilization unit. Make a 1% (v/v) solution of BSA in D-PBS. To this solution, add Type I collagenase to make a 0.1% (w/v) collagenase solution and 1 M calcium chloride to a final concentration of 2 mM.

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- Red cell lysis buffer: Prepare a sterile solution of 155 mM ammonium chloride (NH_4Cl), 10 mM potassium carbonate (K_2CO_3), and 0.1 mM EDTA. Use within two weeks of preparation.

  Freeze medium: Make a solution containing 80% (v/v) FBS, 10% (v/v) DMEM/F12, and 10% (v/v) DMSO.

Cell Differentiation—Incomplete chondrogenic medium. Make up using all reagents listed in Table 2. This medium may be stored at 4° C in the dark for up to 6 weeks.

Complete chondrogenic medium: Thaw growth factors and add freshly to incomplete chondrogenic medium just before use. Three different growth factor combinations have been used successfully to promote ASC chondrogenesis (Table 3).

Alginate Culture—Preparation of 1.2% (w/v) alginate solution: Dissolve 1.2 g of alginate in 100 ml of 150 mM NaCl. Heat on a hot plate and stir thoroughly. Filter sterilize using a 0.22 μm filter. Another option is to prepare the solution using aseptic technique in a sterile hood, thus obviating the need for filter sterilization. Store resulting alginate solution (1.2%) at 4°C.

$CaCl_2$ (102 mM) solution: Place 10.2 ml of a 1 M $CaCl_2$ stock solution into 100 mL volumetric flask and then pipette in 89.8 mL dH_2O. Filter through 250 ml filter system. Store working solution (102 mM) at 4°C.

NaCl (150 mM) solution: Place 15.0 ml of the 1 M NaCl stock solution into 100 mL volumetric flask and then pipette in 85 mL dH_2O. Filter through 250 ml filter system. Store working solution (150 mM) at 4°C.

Sodium Chloride (50 mM) - Sodium Citrate (55 mM) buffer: Dissolve 1.461 g of NaCl into 300 ml of dH_20. Dissolve 8.087 g of sodium citrate into this same solution. Add dH_20 to a final volume of 500 ml.

Sample fixative for histology/immunohistochemistry—Pipette 10 ml of 16% paraformaldehyde into a 50 ml conical tube. Dissolve 0.856 g of sodium cacodylate. If alginate is being fixed, add 0.104 g $BaCl_2$ to irreversibly cross-link the alginate matrix. Titrate the solution with 1 N HCl to a pH of 7.4. Adjust the final volume of the solution to 40 ml with dH_20. Caution: paraformaldehyde and sodium cacodylate are considered carcinogenic. Only handle these reagents in a biosafety level 3 cabinet with the sash adjusted to the proper height to provide maximum protection to the user.

DMB Assay—Preparation of 1,9-dimethlemethylene blue (DMB) dye: Dissolve 21 mg of 1,9-dimethtlemethylene blue into 5 ml of absolute ethanol with 2.0 g of sodium formate and stir thoroughly in 800 ml of distilled water. Titrate concentrated formic acid into the dye solution to adjust the pH to the desired level (pH 1.5 for the alginate DMB assay and pH 3.0 for the pellet cultures). Add distilled water to make a final volume of 1000 ml. This solution may be stored for up to 6 months at room temperature (RT, 19 to 25°C). Note that the solution should be protected from light.

Preparation of Stock Chondroitin-4-Sulfate Standard Solution (10 mg/ml): Weigh 100 mg of chondroitin-4-sulfate (C-4-S). Dissolve the powder in 10 ml of D-PBS in a 50 ml conical tube. Vortex to insure thorough mixing. Dilute 1 ml of the Stock C-4-S solution into 9 ml of Papain solution. This produces a 1 mg/ml C-4-S solution. Freeze in 1 ml aliquots and store at −80°C. Thaw an aliquot on the day of the assay to prepare the standard curve for the DMB assay.

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Hydroxyproline assay—Acetate-Citrate Buffer pH 6.5Dissolve the 12 g of Sodium acetate trihydrate, 5 g of citric acid monohydrate, 1.2 mL acetic acid, and 3.4 g sodium hydroxide in 750 ml of dH_2O. Measure pH and adjust to 6.0 if necessary by titration with 1 N NaOH or acetic acid. Bring the solution to a final volume of 1000 ml with dH_2O. Can be prepared and stored for 1 – 2 months at room temperature.

Chloramine-T Reagent (0.062 M), Dissolve 141 mg of chloramine-T in 2.07 ml of dH_2O and 2.6 ml n-propanol. Note: mix dH_2O and n-propanol first. Add 5.33 ml of the Acetate-Citrate buffer described above. Vortex to dissolve completely. CRITICAL: Must be prepared fresh just before it is to be used.

p-DMBA Reagent (0.94 M), Dissolve 1.4 g of p-dimethylaminobenzaldehyde in 7 ml of n-propanol and vortex. Add 3.0 ml of perchloric acid. Vortex to dissolve completely. CRITICAL: Must be prepared fresh just before it is to be used and used within 1 hour.

Hydroxyproline Standard stock (10 mg/ml), Dissolve 100 mg of *trans*-4 hydroxyproline-L in 10 mL of distilled water to get a stock solution of 10mg/mL. Vortex to dissolve completely. Can be made in advance and stored in 1 ml aliquots at −80°C.

PROCEDURE

ASC Isolation

1 Prepare the water bath shaker at 37 °C, with enough water to cover the bottles containing collagenase mixture.

2 Prepare fresh a solution of 0.1% (w/v) collagenase, 1 % (v/v) bovine serum albumin (fraction V), and 2 mM calcium chloride in PBS. The volume of the collagenase solution should equal the volume of lipoaspirate to be processed (usually 100 to 200 ml).

3 Warm the D-PBS, collagenase solution, and medium to 37 °C.

4 Allow the fat transport container to sit until the fat and blood are well separated. Transfer the fat to a sterile, 250 ml plastic centrifuge tube for separation. Note: cell isolations are routinely performed the day of receipt or performed the next day, after leaving the tissue at room temperature overnight.

CAUTION: Procedures must be performed in accordance with Institutional Review Board policies for obtaining human tissue including informed consent by personnel certified and trained to work with blood borne pathogens. All procedures involving the human tissue should be performed at Biosafety Level 2 with appropriate personal protection.

5 Using a 25 ml serological pipette, aspirate off the blood layer from beneath the floating fat.

6 Check the tissue volume; it should be approximately 100 ml in each 250 ml centrifuge tube.

The isolated cells will be plated at an equivalent to ~35 ml of liposuction aspirate digest per T225 flask so plan the amount of tissue needed accordingly. If there is less tissue volume, the flask size should be reduced accordingly to achieve approximately 0.16 ml tissue per square centimeter.

7 Wash the fat with D-PBS (1:1 volume) up to 7 times or until the color of the D-PBS layer is about the hue of the fat – a light pink color. Allow the two phases to separate between washes (3–5 min). Gently stir the solution while it sits; this

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will encourage separation and aid the washing. Continue to remove the blood layer from beneath the yellow fat tissue. After a few washes, a bright yellow layer of extra cellular fat will begin to form on top of the fat cell layer. Carefully aspirate off this thin layer. Note that 4 washes should be sufficient.

8 After the fat layer has been thoroughly washed, mix it 1:1 (v:v) with the warm D-PBS:collagenase solution. Place it in the 37° C shaker water bath and gently shake for 1 hour. Shake to mix by hand every 20 minutes if using 250 ml centrifuge bottles; or shake to mix by hand every 5–10 minutes if using 50 ml centrifuge tubes.

Note that if a shaker water bath is not available, 50 ml or 250 ml tubes may be placed on a shaker in a 37° C incubator. TROUBLESHOOTING

9 Centrifuge at 300 g at 21° C for 5 minutes.

10 Shake the tubes vigorously for 10 seconds to ensure that individual cells are released from the strands of fibrous tissue.

11 Centrifuge at 300 g at 21° C for 5 minutes.

12 Aspirate off the floating mature adipocyte layer and the aqueous collagenase/D-PBS supernatants, leaving 5 ml of D-PBS on each pellet of cells. Be careful with the cell pellet as it is gelatinous and does not adhere readily to the centrifuge tubes. TROUBLESHOOTING

13 Resuspend each cell pellet in 10 ml stromal medium and pool the suspensions into sterile, 50 ml centrifuge tubes.

14 Centrifuge at 300 g at 21° C for 5 minutes.

15 Aspirate off the supernatants, leaving 5 ml of stromal medium on each pellet.

16 Resuspend cell pellets in 10 ml stromal medium and pool contents into a sterile, 50 ml centrifuge tubes.

17 Centrifuge at 300 g at 21° C for 5 minutes.

18 Aspirate off the supernatants, leaving 5 ml of stromal medium on each pellet.

Note that the pellet can look very different from patient to patient at this stage.

19 Resuspend in a total of 105 ml stromal media per 100 ml of lipoaspirate digest.

TROUBLESHOOTING

ASC Expansion

20 Plate each dish at an equivalent density to 0.16 ml of liposuction aspirate per cm^2 or ~35 ml per 225cm^2 flask.

21 Thoroughly clean up by collecting all contaminated materials into biohazard bags.

22 Clean the hood with 10% detergent, then 10% bleach, then 70% ethanol.

23 After 24 hours, change the media on the cells to wash away any non-adherent cells.

24 Feed the cells every three days for up to 7 days by replacing 100% of the media in each flask.

TROUBLESHOOTING

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25 Harvest cells when they are 80% confluent by trypsinization. Aspirate off expansion medium. Wash the flasks gently with 20 ml of D-PBS warmed to 37° C. Add 8 ml of trypsin/T225 flask, and incubate for 5 minutes at 37 ° C. Verify that the cells have been dislodged from the cell culture plate using a microscope. Inactivate trypsin with an equal amount of stromal medium (i.e., 8 ml).

26 Collect the 16 ml of trypsin/stromal medium in a 50 ml conical tube.

27 Centrifuge at 300 g at 21° C for 5 minutes to pellet cells. Aspirate medium on top of cells.

28 Either passage ASCs further (option A), freeze cells (option B), proceed with chondrogenic differentiation in pellet culture (option C) or proceed with chondrogenic differentiation in alginate beads (option D).

Option A. Further passage. TROUBLESHOOTING

i. Resuspend ASCs in expansion medium and replate at 8,000 cells/cm^2. Representative images of ASCs through 1 passage can be seen in Figure 2. CRITICAL cells may be used up to passage 4, after which the cells will start to lose their differentiation potential.

ii. Ii)Trypisinize cells at ~80% confluence (Figure 2g).

Option B. Freeze cells.

i. Make aliquots of 1–5 million cells/ml in freeze medium in cryovials.

ii. Freeze cells in freeze medium at a rate of −1° C/min using a 1°C freezing container until they reach −80° C, after which the cells may be stored long-term in liquid nitrogen.

PAUSEPOINT Cells can be stored long-term in liquid nitrogen. When thawing cells, place vial in 37° C water bath to thaw cells rapidly. Immediately upon thawing, remove cells from vial and wash cells in 2–3 ml of stromal medium. Centrifuge at 300 g at 21 °C for 5 minutes after which the cells can be resuspended and plated at 8,000 cells/cm^2.

Option C. Chondrogenic differentiation in pellet culture:

i. Following trypsinization of the cells, split desired number of cells (250,000 per pellet) to 15 ml tubes designated for either negative control or chondrogenic conditions.

ii. Resuspend cells in either Incomplete (control) or Complete Chondrogenic Medium at a density of 500,000 cells/mL. Incomplete chondrogenic medium can be used as a negative control. Complete chondrogenic medium with ASCs known to be capable of chondrogenesis may be used as a positive control.

iii. Pipette 0.5 ml of cell suspension into 15 mL polypropylene conical tubes. This yields 250,000 ASCs per tube.

iv. Centrifuge at 300 g at 21 °C for 5 minutes to form a pellet at the bottom of the tube.

Loosen the tops of the conical tubes for gas exchange. Incubate cultures at 37°C and 5% CO_2 overnight.

The following day, the pellets should be rounded in the bottom of the tube.

v. Every other day, for the duration of the experiment, prepare complete chondrogenic medium by adding growth factors and L-Ascorbic acid 2-phosphate and exchange medium. Typical durations for culture are 2, 4, and 6 weeks.

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- **vi.** At each medium exchange, agitate tube gently to ensure the pellet has not adhered to the wall of the tubes.
- **vii.** To harvest, remove the chondrogenic medium and wash the pellets once with D-PBS. Fix the pellet in the paraformaldehyde solution for immunohistochemistry and histology, or digest the pellet in a papain solution for biochemical analysis (see step 29).

Option D. Chondrogenic differentiation in alginate beads:

- **i.** Warm alginate and $CaCl_2$ to 37 °C prior to encapsulation of ASCs.
- **ii.** Resuspend ASCs in 1.2% alginate solution at 5×10^6 cells/ml in a 50 ml conical tube. Mix thoroughly by pipetting without creating bubbles, or mix with the use of micro stir bars using a magnetic stirring plate. Note that if the latter technique is used for mixing, a volume of greater than 700 μl is required to avoid making bubbles.
- **iii.** Using a 1 ml pipette, draw 1 ml of solution into tip.
- **iv.** Dispense by tilting the pipette sideways and slowly pipetting cell suspension such that one drop falls from the pipette tip into 1 ml of pre-warmed $CaCl_2$. Typically, 3 drops of alginate are added to each well of a low attachment surface 24 well plate. (Figure 3 shows a 2.5x image of ASCs encapsulated within alginate.) Note that alginate bead diameter will vary from ~4 – 5mm and will contain approximately 275,000 cells. Five to six wells can be filled per ml of alginate.
- **v.** Incubate the alginate beads at 37°C for 5 minutes to allow Ca^{+2} cations to fully diffuse through the alginate and cross-link the alginate cell suspension.
- **vi.** Pipette off the $CaCl_2$ solution. Note that aspiration can be used, but care must be taken to avoid suction pressure on the alginate beads.
- **vii.** Wash the beads with 1.5 ml incomplete chondrogenic medium at 37°C for 15 minutes. Pipette off incomplete medium and repeat for an additional 15 minutes at 37°C.
- **viii.** Replace incomplete medium with complete chondrogenic medium, 1 ml/well of a 24 well plate. Note that typically 1 ml of medium is used for every 800,000 to 1,000,000 cells. Incomplete chondrogenic medium can be used as a negative control. Complete chondrogenic medium with ASCs known to be capable of chondrogenesis may be used as a positive control.
- **ix.** Incubate at 37°C, 5.0% CO_2. According to experimental design, take samples at day 0 and subsequent time points to be prepared for biochemical examination, histological and immunohistological examination, or gene expression analysis. Accordingly, proceed to the applicable section of this protocol. Refer to Figure 1 for timing for procedures. Every other day for the duration of the experiment, prepare complete chondrogenic medium by adding growth factors and L-Ascorbic acid 2-phosphate and exchange medium. Typical durations for culture are 2, 4, and 6 weeks.

Biochemical determination methods

29 There are a number of options as to how to process the cells further. See Table 1 for information about what each assay assesses. If you wish to perform the dimethyl-methylene blue (DMB) assay[34, 35], follow option A. If you wish to do the hydroxyproline assay[35, 64, 65], follow option B. If you wish to perform histology or immunohistochemistry proceed with option C for all samples

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followed by option D for safranin-o/fast green staining, option E for toluidine blue staining or option F for immunohistochemistry. If you wish to do RT-PCR follow option G.

Option A) Dimethylmethylene blue (DMB) Assay

i. Harvest samples at appropriate time points and digest in 1 ml of papain solution (for alginate beads, 3 beads/well = 1 construct) for 15–18 hours at 65°C. CRITICAL: If wet weight is desired for normalization, weigh the samples before digestion in papain.

PAUSEPOINT If desired, digested samples can be stored at −20°C and then thawed for analysis.

ii. Determine total DNA per construct using the PicoGreen dsDNA quantitation kit per the instructions from the manufacturer. Total dsDNA will be used to normalize DMB content.

iii. Prepare the diluted standard solutions of C-4-S (from 0 to 35 μg/ml) mixing the quantities shown in Table 4 (in microliters) of the 1 mg/ml C-4-S solution with the Papain:

iv. In a clear, flat-bottom 96-well plate, aliquot 40μl of the C-4-S standard solutions and samples to wells. Note that it is likely that some or all samples will need to be diluted with papain at this stage in order for the samples to fall in the range of the standard curve.

v. Add 125 μl of the DMB dye (pH 3.0 for samples not containing alginate) to each well of the plate using a multichannel pipette.

vi. Measure the optical density of the solutions (standard and experimental) using the 595 nm filter (OD_{595}) on a plate reader. Follow option B step

vii. Xxiii onward to calculate S-GAG content.

Option B) Hydroxyproline (OHP) Assay

i. Harvest samples at appropriate time points and digest in 1 ml of papain solution (for alginate beads, 3 beads/well = 1 construct) for 15–18 hours at 65°C. CRITICAL: If wet weight is desired for normalization, weigh the samples before digestion in papain.

PAUSEPOINT If desired, digested samples can be stored at −20°C and then thawed for analysis.

ii. Determine total DNA per construct using the PicoGreen dsDNA quantitation kit per the instructions from the manufacturer. Total dsDNA will be used to normalize hydroxyproline content.

iii. Using pre labeled test tubes, make up your standard solutions by mixing the following proportions of the 1 mg/ml working hydroxyproline solution with D-PBS as follows:

iv. Aliquot 50 μl of the standard solutions and samples in prelabeled microcentrifuge tubes. Note that any dilutions that need to be carried out to bring samples within the range of the standard curve should be performed at this step using papain.

v. Add 50 μl of the 12 N HCl (37%). (Final Concentration ~6N)

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CRITICAL: ensure caps close cleanly without deformation of plastic. Any deformation will result in evaporation of liquid before samples are hydrolyzed.

vi. Hydrolyze the samples by incubating in an oven at 110°C for 15–18 hours (overnight).

TROUBLESHOOTING

vii. Retrieve the tubes from oven.

viii. Spin for 10 or 15 seconds in a microcentrifuge to collect any condensate on the sides and cap of the tubes.

ix. Dry the samples completely either by lyophilization or by a Speedvac. Note that this could take several hours depending on the drying method used. Alternatively, one can remove the caps and place the samples in a laminar flow hood for 2–3 days to dry the samples.

x. Reconstitute the dried samples and standard in 100 μl of the Acetate-Citrate buffer. Mix thoroughly and vortex to dissolve completely.

xi. Add enough activated charcoal to cover the filter chamber of the Spin-X HPLC column. Note that a 1 ml filter tip pipette works well for this purpose.

xii. Place the reconstituted samples and standards in the filter chamber of the Spin-X HPLC microcentrifuge tubes. CRITICAL: Do not discard the tubes as they will be used again in step Xiv

xiii. Spin the samples for 3 minutes at 12,900 g in a microcentrifuge to filter the samples thorough the activated charcoal.

xiv. Add another 100 μl of the Acetate-Citrate buffer to the original tubes (now empty) to collect and reconstitute any residual amounts of the sample. Mix and vortex as before.

xv. Place the reconstituted “residual” samples and standards in the filter chamber of the Spin-X HPLC microcentrifuge tubes.

xvi. Spin again for 3 minutes at 12,900 g in a microcentrifuge to filter the samples thorough the activated charcoal.

xvii. Aliquot triplicates of 50 μl of the samples and standards in individual wells of a 96 well plate.

xviii Add 50 μl of Chloramine-T (0.062 M, prepared fresh as previously described) to the wells. Mix gently on an orbital shaker. Allow the oxidation to proceed for 15 minutes at room temperature.

xix. For chromophore development, add 50 μl of the *p*-DMBA reagent to each sample.

xx. Mix gently on an orbital shaker.

xxi. Allow the reddish/purple color development by incubating the samples at 37°C for 30 minutes.

xxii. Measure the optical density (OD) at 550 (or 540) nm on a plate reader.

xxiii Calculation of the amount of S-GAG or OH-proline: First calculate the average values of the duplicates or triplicates of each of the standard and experimental samples. Use these averages in the following calculations.

xxiv Calculate the “corrected” values of standard optical densities (OD) by taking the difference between the OD for the 0 μg/ml standard and that of the measured

standard. Note that because DMB has a decreasing absorbance with increasing GAG concentration while OHP has increasing absorbance with increasing proline concentration, the terms in the following equations are switched to achieve positive values.

$$S-GAG{:}Corrected\ OD_{x\ \mu g/ml\ standard}=Measured\ OD_{0\ \mu g/ml\ standard}-Measured\ OD_{x\ \mu g/ml\ standard}$$
$$OHP{:}Corrected\ OD_{x\ \mu g/ml\ standard}=Measured\ OD_{x\ \mu g/ml\ standard}-Measured\ OD_{0\ \mu g/ml\ standard}$$

xxv. Calculate the "corrected" values of experimental samples optical densities (OD) by taking the difference between the OD for the 0 μg/ml standard and that of the measured samples

$$S-GAG{:}Corrected\ OD_{Sample}=Measured\ OD_{0\ \mu g/ml\ standard}-Measured\ OD_{Sample}$$
$$OHP{:}Corrected\ OD_{Sample}=Measured\ OD_{sample}-Measured\ OD_{0\ \mu g/ml\ standard}$$

xxvi. Plot the corrected optical density (x-axis) of each of the standard solutions versus the concentration (y-axis) of each of the standard solutions. Use linear regression (forcing a fit through the origin) to obtain the equation describing the linear relationship between the optical density and the concentration.

xxvii. Using linear regression, calculate the concentration of sulfated glycosaminoglycans (GAGs)/OH-proline in the experimental samples based on the corrected $\boldsymbol{OD_{Sample}}$ values. Multiply the calculated concentration by the original sample volume and any dilution factors that were used.

xxviii. For the OH-proline assay, determine the collagen concentration by using the conversion factor of 7.46 mg collagen to 1 mg 4-hydroxyproline. Note that a conversion factor of 10 mg collagen to 1 mg 4-hydroxyproline may be used instead of 7.46 to account for the presence of mostly type II collagen in constructs [66].

xxix. Normalize the results to wet weight of constructs and/or total DNA of constructs. If samples are out of the standard range, dilute the samples appropriately and repeat the measurement, or prepare another standard spanning a wider range.

Option C) Histologic and immunohistochemical methods

i. Immediately following the culture period, place each construct (3 beads = 1 construct for alginate beads) in 20 ml of the paraformaldehyde solution. Fix for 4 hours at RT or overnight at 4°C.

ii. Dehydrate constructs with 30% (v/v) EtOH (diluted in diH_2O) for 30 min, 50% EtOH for 30 min and70% EtOH for 30 min PAUSEPOINT Can be stored long term in 70% ethanol if not continuing immediately.

iii. Dehydrate constructs with 80% EtOH for 30 min, 100% EtOH for 30 min, followed by one additional 100% EtOH wash. PAUSEPOINT. Can be stored overnight in 100% EtOH.

iv. Clear constructs by removing 50% of solution and replacing with xylene yielding a final concentration of 50% EtOH/50% xylene. Incubate 30 min at RT. Replace this 1:1 mixture with 100% xylene and incubate 30 min at RT. Exchange 100% xylene and incubate for an additional 30 min at RT. CRITICAL: ensure that constructs are translucent. If not clear, continue processing with xylene washes until constructs become clear to translucent.

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v. Embed the constructs in paraffin by removing half of the xylene and replacing it with paraffin such that the final concentration is 50% xylene/50% paraffin. Incubate at 60° C for 1 hr. Replace 1:1 mixture with 100% paraffin and incubate at 60° C for 1 hr; replace paraffin again with 100% paraffin and incubate again at 60° C for 1 hr.

CRITICAL: work quickly with the paraffin to ensure that the paraffin does not have time to solidify. If the paraffin solidifies, incubation times must increase to properly infiltrate the construct with paraffin.

vi. Place in embedding tray in desired orientation (attempt to get all 3 alginate beads on the same plane for subsequent sectioning) and allow to harden overnight.

vii. Cut sections 6–10 μm in thickness with a microtome and place in a 45–50° C waterbath.

viii. Place sections on SuperfrostR/Plus Microscope Slides by using slides to remove from waterbath. Allow slides to dry overnight in a 37° C slide warmer. CRITICAL We advise using these particular slides as they have been surface treated for improved adherance of tissue section to slide during processing.

Option D) Safranin-O/fast green

i. Deparaffinize sections in xylene 3 times for 3 minutes each. (Note that for safranin-o/fast green staining, bone, muscles (collagen) are stained green, and cartilage is stained orange or red.)

ii. Re-hydrate in 100% EtOH 2 × 5 minutes, 95% EtOH 1 × 2 minutes, and 70% EtOH 1 × 2 minutes.

iii. Wash in tap water for 30 sec.

iv. Stain in Weigert hematoxylin solution for 8 minutes.

v. Wash in tap water for 3 minutes by repeatedly dipping slides.

vi. Differentiate in Differentiation Solution for 30 seconds.

vii. Wash in tap water for 10 minutes by repeatedly dipping slides (until sections turn a blue hue)

viii. Stain in 0.02 % (w/v) aqueous fast green for 3 minutes.

ix. Rinse for approximately 10 seconds in 1% acetic acid.

x. Dip the slides in tap water briefly and then remove excess water from the slide.

xi. Stain in 0.1 % (v/v) aqueous safranin-O for up to 5 minutes maximum.

xii. Rinse in 100 % EtOH to remove extra red staining on the slide.

xiii. Dehydrate in 95% EtOH 2 × 5 minutes, followed by 100% alcohol 2 × 5 minutes.

xiv. Clear in xylene 3 × 2 minutes.

xv. Mount with Permount.

Option E: Toluidine Blue

i. Re-hydrate in 100% EtOH 2 × 5 minutes, 95% EtOH 1 × 2 minutes, and 70% EtOH 1 × 2 minutes.

ii. Wash in tap water for 30 sec.

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- **iii.** Stain in 0.25 % (w/v in distilled water) aqueous toluidine blue for 5 minutes.
- **iv.** Rinse with distilled water until excess stain is washed away.
- **v.** Dehydrate in 95% EtOH 2 × 5 minutes, followed by 100% alcohol 2 × 5 minutes.
- **vi.** Clear in xylene 3 × 2 minutes.
- **vii.** Mount with Permount.

Option F) Immunohistochemistry (IHC)

- **i.** Deparaffinize by washing slides 3 times × 2 minutes/wash in xylene.
- **ii.** Dip slides in 100% EtOH for 2 washes of 2 minutes each
- **iii.** After slide dries, circle section with PAP pen
- **iv.** Rehydrate slides: 95% EtOH for 2 washes of 2 minutes each; 80% EtOH for 1 wash of 2 minutes; 50% EtOH for 1 wash of 2 minutes; D-PBS for 1 wash of 5 minutes
- **v.** Quench endogenous peroxidase activity by submerging slides in 1 part 30% H_2O_2: 9 parts methanol for 10 minutes.
- **vi.** Wash slides in D-PBS: 3 washes of 2 minutes each
- **vii.** For antigen retrieval, add sufficient Digest-All to completely cover each tissue section
- **viii.** Incubate at RT for 5 minutes
- **ix.** Wash slides in D-PBS: 3 washes of 2 minutes each. (If not labeling for chondroitin sulfate, skip to stepXii)
- **x.** If using antibodies for labeling chondroitin sulfate epitopes, apply chondroitinase ABC to each section and incubate at RT for 20 minutes.
- **xi.** Wash slides in D-PBS: 3 washes of 2 minutes each.
- **xii.** Block sections with Serum Blocking agent (Reagent A in kit)
- **xiii.** Add enough reagent to completely cover tissue section (2–3 drops) TROUBLESHOOTING
- **xiv.** Incubate at RT for 30 minutes (ensure sections do not dry)
- **xv.** Blot excess serum from bottom of inclined slide (do not rinse)
- **xvi.** Dilute primary antibody in non-immune serum, Reagent A, or 10% goat serum (Col I, Col X – 1:400, C-4-S and C-6-S – 1:200, and Col 2 – 1:1).
- **xvii.** Add antibody onto (+) staining sections, and blocking serum onto (−) control sections.
- **xviii** Incubate at room temperature for 1 hour or overnight at 4°C in large plastic petri dish lined with wet filter paper to keep slides moist.
- **xix.** Wash slides in D-PBS: 3 washes of 2 minutes each,
- **xx.** Apply secondary antibody (Reagent B in kit) to cover tissue sections (2 drops).
- **xxi.** Incubate at RT for 10 minutes
- **xxii.** Wash slides in D-PBS: 3 washes of 2 minutes each

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xxiii. Apply enough Enzyme Conjugate (Reagent C in kit) to cover tissue sections (2 drops)

xxiv. Incubate at RT for 10 minutes

xxv. Wash slides in D-PBS: 3 washes of 2 minutes each

xxvi. Add enough Substrate-Chromagen (AEC) mixture to cover tissue (2 drops)

xxvii. Incubate at RT for 20 minutes

xxviii. Gently dip sections in distilled H_2O (leave wet)

xxix. If desired, add 1 2 drops Hematoxylin to counterstain nuclei. If slides are not counterstained, skip to step XXXiii.

xxx. Incubate at RT for 5 minutes

xxxi. Wash with tap H_2O

xxxii. Wash slides in D-PBS for 30s or until slides turn a blue hue.

xxxiii. Wash slides in dH_2O: 3 washes for 2 minutes each (let slides remain in H_2O until coverslips are placed)

xxxiv. Mount slides by applying GVA Mounting Medium to 1 slide at a time and place coverslips.

CRITICAL: do not allow slides to dry before placing mounting medium.

Option G) Real-time quantitative RT-PCR (qPCR) methods

i. For gene expression analysis, first isolate the ASCs from the alginate matrix. Note that gene expression analysis can also be performed on pellets, but the RNA yield is much lower than using alginate beads. For experiments requiring gene expression for pellets, we suggest pooling 5–10 pellets and pulverizing the samples with a mortar and pestle cooled with liquid nitrogen before proceeding with the RNA isolation kit.

ii. Add RNAsecure™ reagent to the sodium chloride, sodium citrate buffer (final concentration, 1X) and distribute to 2 ml microcentrifuge tubes for each construct (3 beads) plus one for temperature control.

iii. Heat solution to 60° C for 10 minutes on a hot plate to activate the RNAsecure™ enzymes.

iv. Cool the solution to 41° C, and add 1 construct (3 beads) to each tube.

v. Agitate tubes every 10 minutes until alginate is in solution. This should take ~ 1 hour. CRITICAL: maintain the solution at 41° C during this entire process to maintain enzyme activity.

vi. Once alginate is no longer visible, centrifuge microcentrifuge tubes at 300 g for 5 minutes at 21° C to pellet cells.

vii. Wash the cells with 500 ml of D-PBS with 1x RNAsecure and centrifuge again for 5 minutes at 21° C to pellet cells.

viii. Aspirate D-PBS.

ix. Either immediately process the cells with the RNA isolation kit or snap freeze in liquid nitrogen and store at −80° C for future processing. PAUSEPOINT Can be stored at −80° C for up to 1 month prior to further processing, though the user is

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cautioned that the samples should be processed as soon as possible to avoid any potential RNA degradation issues. 29. Assess gene expression by following the procedures of Nolan and Bustin [67].

TIMING—Steps 1–19 ASC Isolation (3–5 hours for isolation)

Steps 20–28A ASC Expansion (2–5 weeks for expansion)

Step 28B Freeze Cells (2 Hours)

Step 28C ASC Pellet Culture (2 hours to prepare pellets)

Step 28D ASC Alginate Culture (4 hours to embed ASCs in alginate)

Steps 29A–B Biochemical determination methods (1 hour at appropriate time points to harvest samples and prepare for overnight papain digestion (Step 29). At a later time, dsDNA (Step 30, 2 hours), DMB (Option A, 1 hour), and OHP (Option B, ~10 hours over 2 days)

Steps 29C–F Histologic and immunohistochemical methods (Option C, 1 hour to fix samples overnight, 1 day to embed samples, 15 min/sample for sectioning, several days for staining). Safranin-O/Fast Green (Option D, 1.5 hours), Toluidine Blue (Option E, 1 hour), Immunohistochemistry (Option F, 24 hours).

Step 29G Real-time quantitative RT-PCR (qPCR) methods (2–3 hours at appropriate timepoints to isolate cells and 2 days for subsequent RNA isolation and qPCR at a later time).

ANTICIPATED RESULTS *TROUBLESHOOTING*

ASCs can be expanded rapidly in monolayer culture (Figure 2). Following expansion, ASCs can be differentiated in pellet or alginate culture accordingly, using the induction cocktails listed in Table 3. We have previously defined successful chondrogenesis using histologic and biochemical markers. For pellet culture, one can anticipate successful chondrogenesis as having sulfated glycosaminoglycan content greater than ≥ 931.7 ± 222.3 ng/pellet and having collagen II present in ≥ 29.0 ± 2.2% of the area stained per field of view [37]. This metric was based on histogram-derived thresholds set at values exceeding the 90th percentile of controls for each assay [37]. Similar metrics can also be applied to hydrogel based scaffold systems (e.g., alginate) to discern the degree and success of chondrogenesis; though the reader is reminded that due to the negative charge of the alginate matrix, neither safranin-o nor toluidine blue may be used. Therefore, when using alginate, the user of this protocol must rely on immunohistochemistry to discern the spatial organization of the cartilaginous matrix [45, 51, 56, 57]. Figure 4 demonstrates the variability observed in the accumulation of a sulfated glycosaminoglycan matrix in pellet culture using toluidine blue showing a positive result in 4b; whereas 4c is indicative of a poor or negative result for chondrogenesis. Similarly, under appropriate conditions, ASCs embedded in alginate synthesize collagens and proteoglycan (Figure 5); though negative markers of chondrogenesis (e.g., collagen I and excessive collagen X) should also be monitored. Typically, cartilage-specific matrix molecules are seen most intensely in the pericellular matrix with diffuse staining throughout the tissue-engineered construct. During chondrogenic differentiation, it is also typical to observe a significant size increase in the pellet. This pellet size difference is observed as early as 14 days in culture and persists throughout the duration of the experiment (Figure 6).

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- In terms of biochemical content, typical standard curves for both the DMB and OH-proline assay are seen in Figure 7. In general, we measure S-GAG and OH-proline content in the 4 – 10 μg/μg of dsDNA as we have previously demonstrated [34, 35]. Our qPCR analyses have typically been used to ascertain early chondrogenic events. For example, after 7 days in culture, the addition of 500 ng/ml BMP-6 up-regulated the expression of principal cartilage ECM components, aggrecan (AGC1) and type II collagen (COL2A1) by an average of 205-fold and 38-fold, respectively over day-0 controls, while down-regulating the expression of type X collagen (COL10A1) expression by approximately 2-fold [45]; though it should be noted that qPCR data must be evaluated in concert with the other assays detailed in this protocol to determine chondrogenic efficiency. This is also true, in general, as the achievement of a specific value or threshold, in terms of biochemical content or fold-difference over control through qPCR analyses, does not necessarily indicate successful chondrogenesis. The degree and success of chondrogenic differentiation must ultimately be assessed by the investigator when viewed in terms of the hypothesis, objectives, the chondrogenic culture system, and the sum total of all assays employed to assess chondrogenesis.

Troubleshooting—See Troubleshooting guidance in table 6.

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References

1. Hootman JM, Brault MW, Helmick CG, Theis KA, Armour BS. Prevalence and most common causes of disability among adults United States, 2005. Morbidity and Mortality Weekly Report. 2009; 58:421–426. [PubMed: 19407734]
2. Praemer, A.; Furner, S.; Rice, DP. Musculoskeletal Conditions in the United States. American Academy of Orthopaedic Surgeons; Rosemont, IL: 1999.
3. Goldring MB. Update on the biology of the chondrocyte and new approaches to treating cartilage diseases. Best Pract Res Clin Rheumatol. 2006; 20:1003–25. [PubMed: 16980220]
4. Jackson DW, Simon TM, Aberman HM. Symptomatic articular cartilage degeneration: the impact in the new millennium. Clin Orthop Relat Res. 2001:S14–25. [PubMed: 11603698]
5. Rotter N, Haisch A, Bucheler M. Cartilage and bone tissue engineering for reconstructive head and neck surgery. Eur Arch Otorhinolaryngol. 2005; 262:539–45. [PubMed: 16091977]
6. Langer R, Vacanti JP. Tissue engineering. Science. 1993; 260:920–6. [PubMed: 8493529]
7. Aichroth PM, Patel DV, Moyes ST. A prospective review of arthroscopic debridement for degenerative joint disease of the knee. Int Orthop. 1991; 15:351–5. [PubMed: 1809718]
8. Aubin PP, Cheah HK, Davis AM, Gross AE. Long-term followup of fresh femoral osteochondral allografts for posttraumatic knee defects. Clin Orthop Relat Res. 2001:S318–27. [PubMed: 11603715]
9. Baumgaertner MR, Cannon WD Jr, Vittori JM, Schmidt ES, Maurer RC. Arthroscopic debridement of the arthritic knee. Clin Orthop Relat Res. 1990:197–202. [PubMed: 2317974]
10. Denoncourt PM, Patel D, Dimakopoulos P. Arthroscopy update #1. Treatment of osteochondrosis dissecans of the knee by arthroscopic curettage, follow-up study. Orthop Rev. 1986; 15:652–7. [PubMed: 3453907]
11. Emmerson BC, et al. Fresh osteochondral allografting in the treatment of osteochondritis dissecans of the femoral condyle. Am J Sports Med. 2007; 35:907–14. [PubMed: 17369560]

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12. Friedman MJ, Berasi CC, Fox JM. Preliminary results with abrasion arthroplasty in the osteoarthritic knee. Clinical Orthopaedics and Related Research NO. 1984; 182:200–205.
13. Ghazavi MT, Pritzker KP, Davis AM, Gross AE. Fresh osteochondral allografts for post-traumatic osteochondral defects of the knee. J Bone Joint Surg Br. 1997; 79:1008–13. [PubMed: 9393922]
14. Johnson LL. Arthroscopic abrasion arthroplasty: a review. Clin Orthop Relat Res. 2001:S306–17. [PubMed: 11603714]
15. Kish G, Modis L, Hangody L. Osteochondral mosaicplasty for the treatment of focal chondral and osteochondral lesions of the knee and talus in the athlete. Rationale, indications, techniques, and results. Clin Sports Med. 1999; 18:45–66. vi. [PubMed: 10028116]
16. Steadman JR, et al. Outcomes of microfracture for traumatic chondral defects of the knee: average 11-year follow-up. Arthroscopy. 2003; 19:477–84. [PubMed: 12724676]
17. Steadman JR, Rodkey WG, Rodrigo JJ. Microfracture: surgical technique and rehabilitation to treat chondral defects. Clin Orthop Relat Res. 2001:S362–9. [PubMed: 11603719]
18. Nehrer S, Spector M, Minas T. Histologic analysis of tissue after failed cartilage repair procedures. Clin Orthop Relat Res. 1999:149–62. [PubMed: 10627699]
19. Shapiro F, Koide S, Glimcher MJ. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. J Bone Joint Surg Am. 1993; 75:532–53. [PubMed: 8478382]
20. Tew SR, Kwan AP, Hann A, Thomson BM, Archer CW. The reactions of articular cartilage to experimental wounding: role of apoptosis. Arthritis Rheum. 2000; 43:215–25. [PubMed: 10643718]
21. Breinan HA, et al. Effect of cultured autologous chondrocytes on repair of chondral defects in a canine model. J Bone Joint Surg Am. 1997; 79:1439–51. [PubMed: 9378730]
22. Brittberg M, et al. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med. 1994; 331:889–95. [PubMed: 8078550]
23. Knutsen G, et al. Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial. J Bone Joint Surg Am. 2004; 86-A:455–64. [PubMed: 14996869]
24. Lee CR, Grodzinsky AJ, Hsu HP, Martin SD, Spector M. Effects of harvest and selected cartilage repair procedures on the physical and biochemical properties of articular cartilage in the canine knee. Journal of Orthopaedic Research. 2000; 18:790–799. [PubMed: 11117302]
25. Thirion S, Berenbaum F. Culture and phenotyping of chondrocytes in primary culture. Methods Mol Med. 2004; 100:1–14. [PubMed: 15280583]
26. Stokes DG, et al. Assessment of the gene expression profile of differentiated and dedifferentiated human fetal chondrocytes by microarray analysis. Arthritis Rheum. 2002; 46:404–19. [PubMed: 11840443]
27. Ohara K, Nakamura K, Ohta E. Chest wall deformities and thoracic scoliosis after costal cartilage graft harvesting. Plast Reconstr Surg. 1997; 99:1030–6. [PubMed: 9091899]
28. Chang SC, Tobias G, Roy AK, Vacanti CA, Bonassar LJ. Tissue engineering of autologous cartilage for craniofacial reconstruction by injection molding. Plast Reconstr Surg. 2003; 112:793–9. discussion 800-1. [PubMed: 12960860]
29. Caplan AI, Bruder SP. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med. 2001; 7:259–64. [PubMed: 11378515]
30. Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp. 1988; 136:42–60. [PubMed: 3068016]
31. Pittenger MF, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999; 284:143–7. [PubMed: 10102814]
32. Aust L, et al. Yield of human adipose-derived adult stem cells from liposuction aspirates. Cytotherapy. 2004; 6:7–14. [PubMed: 14985162]
33. Gimble JM, Guilak F. Differentiation potential of adipose derived adult stem (ADAS) cells. Curr Top Dev Biol. 2003; 58:137–60. [PubMed: 14711015]
34. Awad HA, Halvorsen YD, Gimble JM, Guilak F. Effects of transforming growth factor beta1 and dexamethasone on the growth and chondrogenic differentiation of adipose-derived stromal cells. Tissue Eng. 2003; 9:1301–12. [PubMed: 14670117]

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35. Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials. 2004; 25:3211–22. [PubMed: 14980416]
36. Erickson GR, et al. Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. Biochem Biophys Res Commun. 2002; 290:763–9. [PubMed: 11785965]
37. Guilak F, et al. Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. J Cell Physiol. 2006; 206:229–37. [PubMed: 16021633]
38. Halvorsen YC, Wilkison WO, Gimble JM. Adipose-derived stromal cells--their utility and potential in bone formation. Int J Obes Relat Metab Disord. 2000; 24 (Suppl 4):S41–4. [PubMed: 11126240]
39. Huang CY, Reuben PM, D'Ippolito G, Schiller PC, Cheung HS. Chondrogenesis of human bone marrow-derived mesenchymal stem cells in agarose culture. Anat Rec A Discov Mol Cell Evol Biol. 2004; 278:428–36. [PubMed: 15103737]
40. Safford KM, et al. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun. 2002; 294:371–9. [PubMed: 12051722]
41. Wickham MQ, Erickson GR, Gimble JM, Vail TP, Guilak F. Multipotent stromal cells derived from the infrapatellar fat pad of the knee. Clin Orthop Relat Res. 2003:196–212. [PubMed: 12838072]
42. Zuk PA, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002; 13:4279–95. [PubMed: 12475952]
43. Zuk PA, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001; 7:211–28. [PubMed: 11304456]
44. Wang DW, Fermor B, Gimble JM, Awad HA, Guilak F. Influence of oxygen on the proliferation and metabolism of adipose derived adult stem cells. J Cell Physiol. 2005; 204:184–91. [PubMed: 15754341]
45. Estes BT, Wu AW, Guilak F. Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. Arthritis Rheum. 2006; 54:1222–32. [PubMed: 16572454]
46. Oedayrajsingh-Varma MJ, et al. Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure. Cytotherapy. 2006; 8:166–77. [PubMed: 16698690]
47. Estes BT, Diekman BO, Guilak F. Monolayer cell expansion conditions affect the chondrogenic potential of adipose-derived stem cells. Biotechnol Bioeng. 2008; 99:986–95. [PubMed: 17929321]
48. Hennig T, et al. Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6. J Cell Physiol. 2007; 211:682–91. [PubMed: 17238135]
49. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res. 1998; 238:265–72. [PubMed: 9457080]
50. Cheng NC, Estes BT, Awad HA, Guilak F. Chondrogenic Differentiation of Adipose-Derived Adult Stem Cells by a Porous Scaffold Derived from Native Articular Cartilage Extracellular Matrix. Tissue Engineering Part A. 2009; 15:231–241. [PubMed: 18950290]
51. Diekman BO, Rowland CR, Caplan AI, Lennon D, Guilak F. Chondrogenesis of adult stem cells from adipose tissue and bone marrow: Induction by growth factors and cartilage derived matrix. Tissue Eng Part A. 200910.1089/ten.TEA.2009.0398
52. Guilak, F.; Hung, CT. Basic Orthopaedic Biomechanics and Mechano-Biology. Mow, VC.; Huiskes, R., editors. Lippincott Williams & Wilkins; Philadelphia: 2004.
53. Mow VC, Guo XE. Mechano-electrochemical properties of articular cartilage: their inhomogeneities and anisotropies. Annu Rev Biomed Eng. 2002; 4:175–209. [PubMed: 12117756]
54. Mow, VC.; Proctor, CS.; Kelly, MA. Basic Biomechanics of the Musculoskeletal System. Nordin, M.; Frankel, VH., editors. Lea & Febiger; Philadelphia, London: 1989. p. 31-58.
55. Heinegård D, Oldberg A. Structure and biology of cartilage and bone matrix noncollagenous macromolecules. FASEB Journal. 1989; 3:2042–51. [PubMed: 2663581]

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56. Diekman BO, Estes BT, Guilak F. The effects of BMP6 overexpression on adipose stem cell chondrogenesis: Interactions with dexamethasone and exogenous growth factors. J Biomed Mater Res A. 200910.1002/jbm.a.32589
57. Estes BT, Wu AW, Storms RW, Guilak F. Extended passaging, but not aldehyde dehydrogenase activity, increases the chondrogenic potential of human adipose-derived adult stem cells. J Cell Physiol. 2006; 209:987–95. [PubMed: 16972251]
58. Pilgaard L, et al. Effect of oxygen concentration, culture format and donor variability on in vitro chondrogenesis of human adipose tissue-derived stem cells. Regen Med. 2009; 4:539–48. [PubMed: 19580403]
59. Vidal MA, et al. Comparison of chondrogenic potential in equine mesenchymal stromal cells derived from adipose tissue and bone marrow. Vet Surg. 2008; 37:713–24. [PubMed: 19121166]
60. Mehlhorn AT, et al. Differential effects of BMP-2 and TGF-beta1 on chondrogenic differentiation of adipose derived stem cells. Cell Prolif. 2007; 40:809–23. [PubMed: 18021172]
61. Guilak F, et al. Control of stem cell fate by physical interactions with the extracellular matrix. Cell Stem Cell. 2009; 5:17–26. [PubMed: 19570510]
62. Moutos FT, Freed LE, Guilak F. A biomimetic three-dimensional woven composite scaffold for functional tissue engineering of cartilage. Nat Mater. 2007; 6:162–7. [PubMed: 17237789]
63. Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. Mol Cell Biol. 1997; 17:2336–46. [PubMed: 9121483]
64. Neidert MR, Lee ES, Oegema TR, Tranquillo RT. Enhanced fibrin remodeling in vitro with TGF-beta1, insulin and plasmin for improved tissue-equivalents. Biomaterials. 2002; 23:3717–31. [PubMed: 12109697]
65. Stegemann H, Stalder K. Determination of hydroxyproline. Clin Chim Acta. 1967; 18:267–73. [PubMed: 4864804]
66. Hollander AP, et al. Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. J Clin Invest. 1994; 93:1722–32. [PubMed: 7512992]
67. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat Protoc. 2006; 1:1559–82. [PubMed: 17406449]

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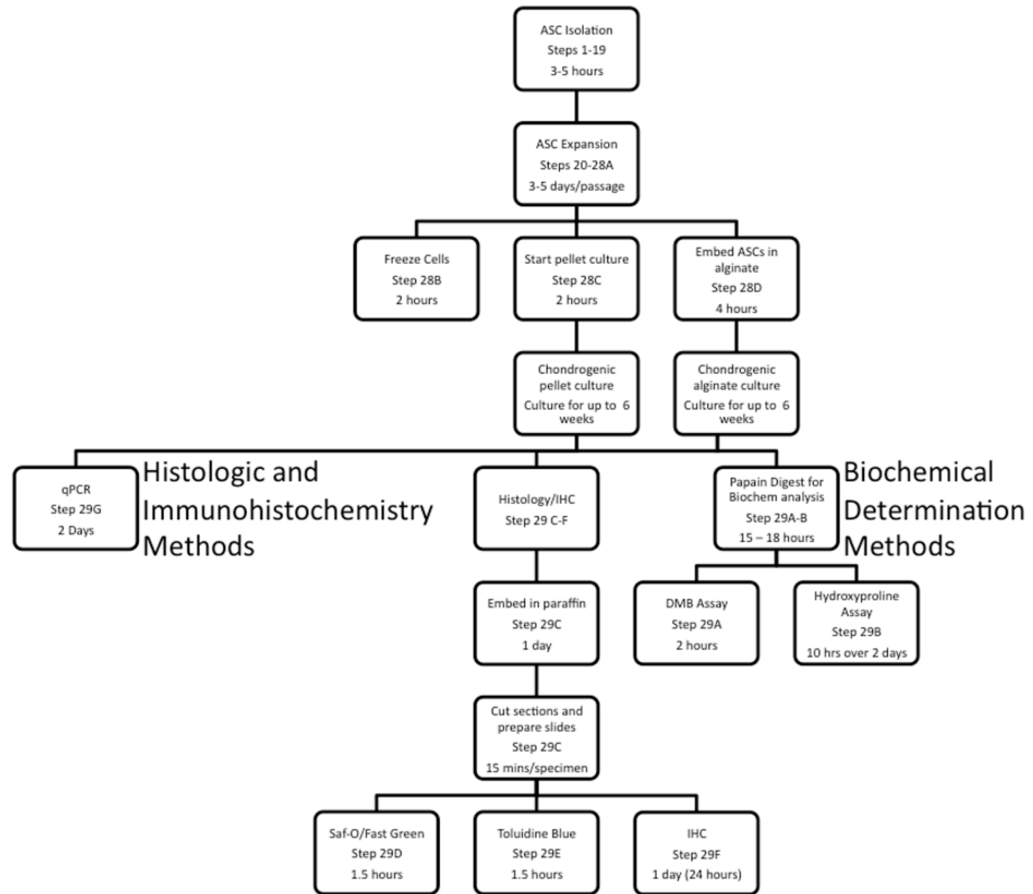

Figure 1.
Flow chart for experiments with associated timing

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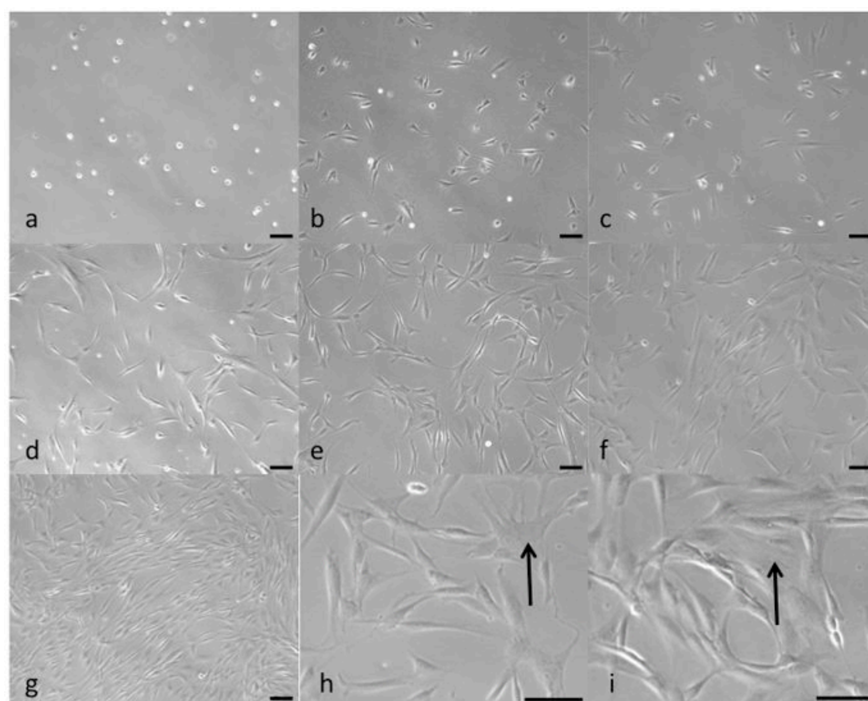

Figure 2.

Expansion of ASCs in expansion medium over 5 days. Appearance of cells at 10x after (a) 30 minutes, (b) 3 hours, (c) 1 day, (d) 2 days, (e) 3 days, (f) 4 days, and (g) 5 days. Black arrows in (h) and (i) show cells with atypical or abnormal morphology. Scale bar = 100 mm.

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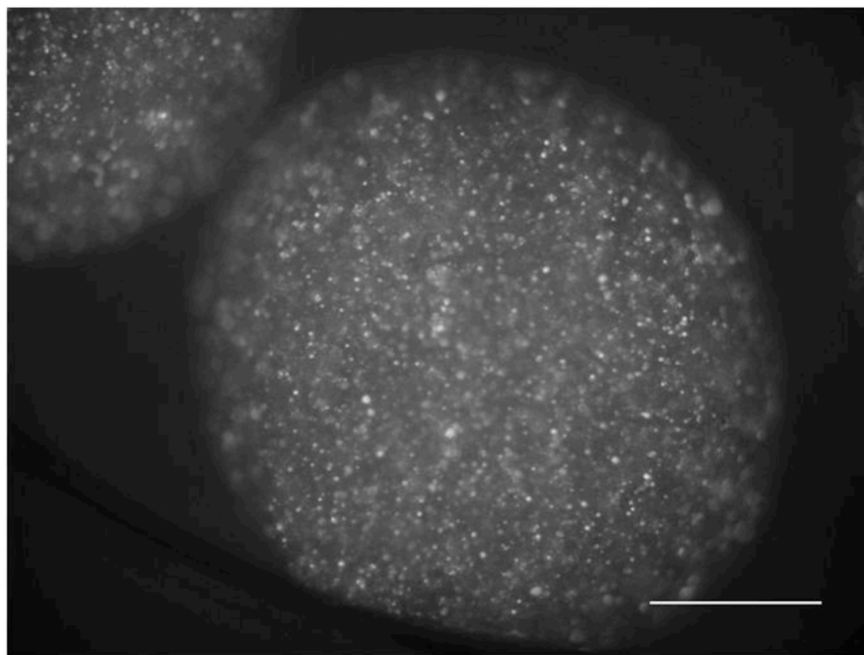

Figure 3.
2.5x microscope image (2.5x) of ASCs encapsulated in alginate. Scale bar = 1 mm.

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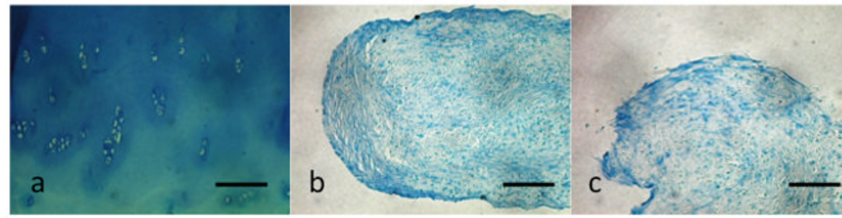

Figure 4.
Toluidine Blue stain on (a) human articular cartilage and (b) and (c) are representative images of typical variation in GAG synthesis and accumulation obtained from ASC pellets cultured for 14 days. (Note. Expect similar results if Safranin-O stain were used.) Scale bars = 100 mm.

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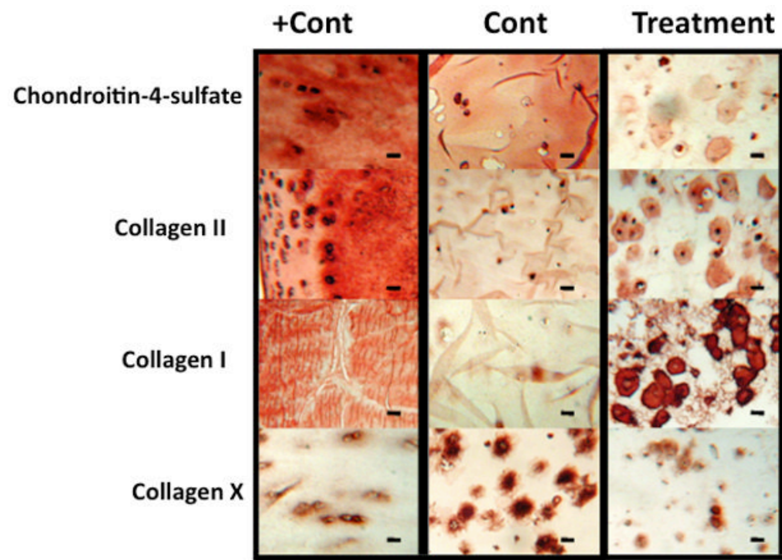

Figure 5.
Representative immunohistochemistry results for chondroitin-4-sulfate and types I, II, and X collagen for a typical experiment with ASCs encapsulated in alginate after 4 weeks in in vitro culture. Positive Control: porcine cartilage for C-4-S, Collagen II, and Collagen X. Porcine ligament for Collagen 1. Control: incomplete chondrogenic medium supplemented with 10% FBS. Treatment: incomplete chondrogenic medium supplemented with short term exposure to BMP-6 in addition to continuous exposure to rhEGF, rhFGF, and 10% FBS. Scale bars = 20 mm. Figure modified from Diekman, B.O., Estes, B.T. & Guilak, F. The effects of BMP6 overexpression on adipose stem cell chondrogenesis: Interactions with dexamethasone and exogenous growth factors. *J Biomed Mater Res A (2009)*. Reprinted with permission from John Wiley and Sons.

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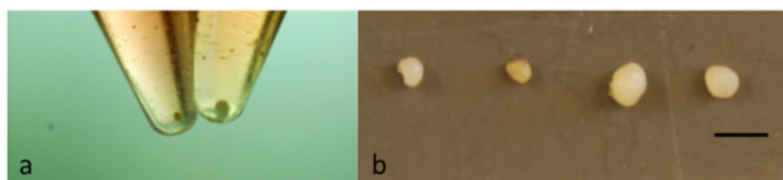

Figure 6.

Pellet size after 6 weeks of culture. (a) representative image of pellets in 15 ml conical tubes in incomplete chondrogenic medium + 10% FBS on the left and complete chondrogenic medium containing TGF-b on the right and (b) the left two pellets were cultured in incomplete chondrogenic medium + 10% FBS and the right two pellets were cultured in complete chondrogenic medium containing TGF-b. Scale bar = 1 mm.

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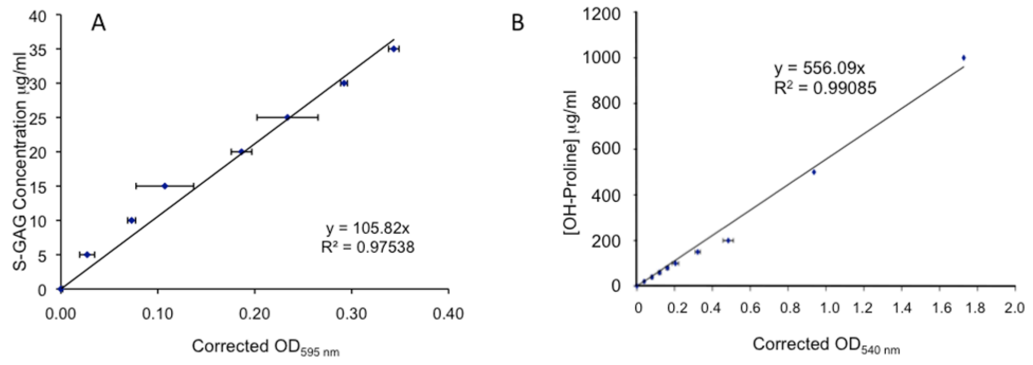

Figure 7.
Typical DMB (A) and OH-Proline (B) assay standard curves. n=3 wells per concentration. Data are mean values ± SEM.

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Table 1

Assaysto determine ASC chondrogenesis

Assay	Utility
Dimethyl-Methylene Blue Assay (DMB)	Determines sulfated glycosaminoglycan content, a critical extracellular matrix component of articular cartilage
Hydroxyproline Assay (OHP)	Determines amount of synthesizedcollagen. Type II collagen is the structural matrix protein in articular cartilage that resists tensile forces, making it a critical extracellular protein component.
Double Stranded Deoxyribonucleic Acid content (dsDNA)	Used to determine cell viabilityand normalize the DMB and OHP assay results.
Safranin-O/Fast Green and Toluidine Blue Histological Stain	Displays the spatial organization of the negatively charged proteoglycan along with the collagenous matrix. Note: These stains cannot be performed on alginate sections due to its negative charge.
Type II collagen immunohistochemistry (IHC) Type I collagen immunohistochemistry Type X collagen immunohistochemistry Chondroitin 4-sulfate Chondroitin 6-sulfate	Predominant type II cartilage indicates a chondrocyte-like phenotype. Predominant type I cartilage indicates a fibrous matrix. Type X collagen is considered as a marker of hypertrophic chondrocytes undergoing terminal differentiation. Chondroitin 4-sulfate and 6-sulfate glycosaminoglycans are found on aggrecan side chains in articular cartilage. The negative charge of these molecules contributes to binding of water and resisting high compressive forces.
Quantitative PCR (qPCR)	Enables an analysis of keycartilage transcription factors and extracellular matrix proteins, and provides early data on the differentiation profile of the ASCs.

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Table 2

TaqMan Assays on Demand

Assay ID	GenBank mRNA Sequence	Gene Symbol	Purpose
Hs00153936_m1	M55172.1	AGC1	Aggrecan is a large aggregating proteoglycan found in articular cartilage. High levels of AGC1 transcript are desirable.
Hs00164004_m1	M36546.1	COL1A1	Type I collagen is highly expressed in ASCs. Monitoring COL1A1 transcript reveals the degree to which fibrous tissue may develop.
Hs00166657_m1	BI521533.1	COL10A1	Type X collagen is the hallmark hypertrophic chondrocytemarker. Type X collagen is found in the transition from articular cartilage to bone; so some level of COL10A1 is desirable. Excessive expression may indicate tissues tending toward an ossification pathway.
Hs00156568_m1	BC116449.1	COL2A1	Type II collagen dominates the extracellular matrix of articular cartilage and provides a critical mechanical function. Thus high levels of COL2A1 are desirable.
Hs00165814_m1	Z46629.1	SOX9	SOX9 is a potent chondrogenic transcription factor and is closely correlated with COL2A1 transcription(67).
Hs99999901_s1	X03205.1	18S	18S is used as a housekeeping gene.

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Table 3

Media components for expanding ASCs

Component	Stock Conc.	Volume for 500 ml bottle	Final Conc.
DMEM/F12	100%	444.89	~89%
Fetal Bovine Serum	100%	50 ml	10%
Human epidermal growth factor (rhEGF)	50 μg/ml	50 μl	5 ng/ml
Recombinant Human fibroblastic growth factor, basic (rhFGF)	10 μg/ml	50 μl	1 ng/ml
Transforming growth factor β1 (TGF-β1), purified from human platelets	10 μg/ml	12.5 μl	0.25 ng/ml
Antibiotic/Antimycotic (Pen/Strep/Fung)	100×	5 ml	1× (100 μg/ml streptomycin, 100 U/ml penicillin, 250 ng/ml Fungizone)

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Table 4

Incomplete chondrogenic medium

Component	Stock Conc.	Dilution	Final Conc.
DMEM HG	100%	n/a	~89%
Fetal Bovine Serum	100%	1:10	10%
ITS +	100×	1:100	6.25 μg/mL bovine insulin 6.25 μg/mL transferrin 6.25 μg/mL selenous acid 5.33 μg/mL linoleic acid 1.25 mg/mL BSA
Dexamethasone	10 mM	1:100	100 nM
Penicillin/Streptomycin	100×	1:100	1× (100 μg/ml streptomycin, 100 U/ml penicillin)

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Table 5

Growth Factor Combinations for ASC chondrogenesis*

Options	Growth Factors	Stock Conc.	Dilution	Final Conc.
1(refs 34,36, 43)	TGF-β1	10 μg/ml	1:1000	10 ng/ml
2(refs 45, 57)	BMP-6	50 μg/ml	1:100	500 ng/ml
3(ref 48)	TGF-β3	10 μg/ml	1:1000	10 ng/ml
	BMP-6	10 μg/ml	1:1000	10 ng/ml

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Table 6

Volumes for chondroitin 4-sulfate (C-4-S) standard curve

Concentration of C-4-S (μg/ml)	Volume of 1 mg/ml C-4-S (μl)	Volume of papain (μl)
0	0	1000
5	5	995
10	10	990
15	15	985
20	20	980
25	25	975
30	30	970
35	35	965

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Table 7

Hydroxyproline standards

OHP Concentration (μg/ml)	D-PBS (μl)	OHP (μl)
0	1000	0
20	980	20
40	960	40
60	940	60
80	920	80
100	900	100
150	850	150
200	800	200
300	700	300
500	500	500
1000	0	1000

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Table 8

Troubleshooting table.

Step or Section	Problem	Possible Reason	Possible Solution
Table 5, Option 3	Variability with induction efficiency.	Results may be serum dependent as differentiation is completed in the presence of 10% FBS (51).	Screendifferent lots of serum for chondrogenic effectiveness and/or investigate serum-free solutions (bone marrow-derived MSCs are often chondrogenically differentiated without serum)
Step 8	Collagenase digestion is not effective.	The characteristics of collagenase can vary between lots provided by the same commercial source. As a result, incomplete digestion can occur when a new lot of collagenase or different vendor are used.	It is important to perform quality control analyses of each collagenase lot prior to purchase and most vendors will accommodate your requirement for such testing. If the source of collagenase does not address the problem, the digestion period can be extended to between 90–120 minutes.
Step 12	The pellet is “contaminated” with red blood cells.	Even with extensive PBS washes, it is not uncommon to find a significant number of erythrocytes in the stromal vascular fraction pellet.	These will not interfere with the culture of the ASC, per se. Nevertheless, it is possible to remove theerythrocytes by resuspending the pellet in a red cell lysis buffer for up to 5 minutes followed by a wash of the cells in stromal medium.
Step 19	The pellet contains tissue fragments, not just a single cell suspension.	Although most of the extracellular matrix has been degraded, some collagenous material remains.	The presence of the tissue fragments will not interfere with the cell isolation; indeed, stromal/stem cells will migrate from the tissue fragments during the initial period of culture. Nevertheless, it is possible to remove the tissue fragments by passing the re-suspended stromal vascular fraction through a 100 micron filter before seeding in the 225 cm^2 flask.
Step 24, 28A	The cells grow too slowly.	Cell proliferation varies between donors. Nevertheless, growth to confluence that exceeds 7 days is problematic.	The cell doubling time can be increased dramatically (> 2 population doublings in 2–3 days) using expansion medium supplemented with basic fibroblast and epidermal growth factors and transforming growth factor β. Indeed, some laboratories use expansion medium exclusively during the early passage of the cells. The cells should also maintain thin, spindle-shaped cell bodies during expansion. The user should consider the use of expansion medium should the cells stop dividing and/or start to spread out on the plates and lose their spindle-shaped appearance.
Step 28A	Cells spread out on plate and stop growing. Cells appear like what is shown in figure 2h and 2i.	This behavior can be observed if the user attempts to expand the cells through more than 4 passages, specifically if expansion medium is not used.	Start with new cells or cells previously frozen from this lot of cells and expand the cells in the presence of expansion medium. Switching to expansion medium from stromal medium should resolve this problem.
Step 41	Tubes are empty after hydrolysis or a significant volume has evaporated.	The caps were not properly snapped on the tubes.	Measure the volume of the samples to ensure that extensive evaporation did not occur (should recover > 90 µl of original 100 µl). If evaporation is observed, an alternative is to use cryovials wrapped tightly with Teflon tape.
Step 97	Non-specific labeling of immunohistochemistry sections.	Most immunohistochemistry labeling kits provide a broad spectrum secondary antibody, which may result in non-specific labeling.	Antibody specificity may be increased if necessary by using an alternate secondary antibody (Sigma, B7151) diluted 1:400 in D-PBS with 1% (v/v) BSA.
Anticipated results	ASCs do not differentiate well.	The degree of chondrogenic potential of ASCs may be dependent on the specific donor(45, 57).	Different donors may be screened for chondrogenic potential. It is also possible that the ASCs may respond more favorably to a different chondrogenic cocktail (Table 5).