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Three-dimensional Culture Systems to Induce Chondrogenesis of Adipose-Derived Stem Cells

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Summary

Stem cells can easily be harvested from adipose tissue in large numbers for use in tissue engineering approaches for cartilage repair or regeneration. In this chapter, we describe in vitro tissue engineering models that we have used in our laboratory for the chondrogenic induction of adipose-derived stem cell (ASC). In addition to the proper growth factor environment, chondrogenesis requires cells to be maintained in a rounded morphology in three-dimensional (3D) culture, and thus properties of the biomaterial scaffold also play a critical role in ASC differentiation. Histologic and immunohistologic methods for assessing chondrogenesis are also presented. In general, 10–12 weeks are required to assess ASC chondrogenesis in these model systems.

Keywords

adipose-derived stem cells (ASC); chondrogenesis; tissue engineering; alginate; agarose; pellet culture

1. INTRODUCTION

Damage to articular cartilage, either through trauma or degenerative joint disease, poses tremendous challenges to the medical community, as cartilage exhibits little to no intrinsic repair capabilities. Much effort has gone into the development of methods to promote regeneration and/or repair of the damage tissue through microfracture of the subchondral bone, abrasion arthroplasty, or the transplantation of autologous or allogeneic osteochondral grafts (1–11). While the early results from these clinical procedures have been somewhat encouraging, complete regeneration and repair of the cartilage has yet to be achieved, and long-term outcomes show little difference between the microfracture technique and autologous chondrocyte implantation (12). The most common form of arthritis is osteoarthritis, which results in a degeneration of the articular surfaces of cartilage. With the direct and indirect costs of treatment of osteoarthritis escalating to greater than \$65 billion annually (13), approaches that result in regeneration of cartilage and improved patient outcomes are welcomed. To this end, one promising approach for cartilage tissue regeneration is through a tissue engineering approach employing adipose derived stem cells (ASCs), as these cells can be autologously harvested in large numbers and have demonstrated the ability by many investigators to be capable of chondrogenic differentiation (14–25). This protocol presents methods that have been used by us and others to induce chondrogenesis of ASCs in 3D culture systems using either pellet culture or encapsulation in

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hydrogels such as agarose or alginate. These culture systems maintain the cells in a rounded shape, which appears to be critical for chondrogenesis and prevention of a fibroblastic phenotype.

2. MATERIALS

2.1 Cells

Adipose derived stem cells derived from subcutaneous adipose liposuction aspirates (see Chapter 2).

2.2 Supplies

Alginate bead culture

- Alginate
- Sodium Chloride (NaCl)
- Sodium Citrate Trisodium salt dihydrate
- Calcium Chloride
- Sterile Syringe filter (0.22 μ m)
- 150 ml 0.22 μ m filter system
- 50 mL polypropylene centrifuge tubes, sterile
- 24 well plate, with lid, flat bottom, Ultra-low attachment surface
- Micro stir bars (12.7 mm $\times$ 3 mm)

Agarose culture

- Type VII low melting point agarose
- Phosphate Buffered Saline
- Sterile Syringe filter (0.22 μ m)
- 15 mL polypropylene centrifuge tubes, sterile
- 24 well plate, with lid, flat bottom, Ultra-low attachment surface
- Micro stir bars (12.7 mm $\times$ 3 mm)
- Multiple needles gauges for drawing and dispensing agarose
- Mold for agarose gel – Note: molds vary from a simple petri dish, wells of a specific size plate, or a gel tray to a complex custom-designed mold with a more complex, or specifically defined shape for its intended purposes (26–28).
- Skin biopsy punches of desired size (used for punching specific sized constructs for culture)

Pellet culture

- 15 mL polypropylene centrifuge tubes, sterile

Chondrogenic Induction

- Dulbecco's Modified Eagles Medium-high glucose, (DMEM-HG)
- ITS+ supplement

- Dexamethasone
- L-Ascorbic acid 2-phosphate Sesquimagnesium Salt
- Penicillin/Streptomycin
- Transforming Growth Factor beta-3 (TGF- β 3)
- Bone Morphogenetic Protein-6 (BMP-6)
- Siliconized 200 μ l Pipette tips
- Siliconized 0.6 mL Snap-Cap microtubes
- 15 mL polypropylene centrifuge tubes, sterile
- 50 mL polypropylene centrifuge tubes, sterile

dsDNA Quantitation

- PicoGreen dsDNA Quantitation kit (Invitrogen) or equivalent

Fixation

- 16% paraformaldehyde
- Sodium Cacodylate, Trihydrate
- Barium chloride

Histology

- Xylene
- Tissue Embedding Medium (Paraffin)
- Histology cassettes
- Microscope slides
- Safranin-O
- Fast Green FCF
- Weigert Hematoxylin Solution
- Differentiation solution
- Mounting solution

Immunohistochemistry

- Immunohistochemical staining kit
- Type II collagen antibody
- Type I collagen antibody
- Type X collagen antibody
- 2-B-6 Chondroitin-4-Sulfate antibody
- 3-B-3 Chondroitin-6-Sulfate antibody
- Xylene
- AEC Substrate-Chromagen

- Pepsin
- Chondroitinase ABC
- PAP Pen
- Methanol
- Hydrogen Peroxide Goat serum
- GVA Mounting Medium (Invitrogen) or equivalent

2.3 Equipment

- Centrifuge
- Water bath shaker
- Microplate reader (for fluorescence based dsDNA quantitation)
- Microtome
- Hot plate with temperature control

2.4 Stock solutions

Cell Differentiation—Incomplete chondrogenic medium (Table 1). This medium may be stored at 4°C in the dark for up to 6 weeks.

Complete chondrogenic medium: growth factors are thawed and added freshly to incomplete chondrogenic medium just before use. Three different growth factor combinations have been used successfully to promote ASC chondrogenesis (Table 2).

Alginate Culture—Prepare 1.2% (w/v) alginate solution by dissolving alginate into 150 mM NaCl. Heat on a hot plate and stir thoroughly. Care should be taken that the solution is not boiled or that it is not overly heated such that excess evaporation occurs. 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.

CaCl₂ (102 mM) solution: Prepare solution in dH₂O, and filter sterilize using a 0.22 µm filter. Store working solution (102 mM) at 4°C.

NaCl (150 mM) solution: Prepare solution in dH₂O and filter sterilize using a 0.22 µm filter. Store working solution (150 mM) at 4°C.

Agarose Culture—Prepare a 2% (w/v) agarose solution by dissolving type VII agarose in D-PBS. This solution can be made on a hot plate or in the microwave. Care should be taken to not allow excess evaporation to take place during this process. Once the agarose is in solution, quickly filter sterilize using a 0.22 µm syringe filter. Note that if excessive cooling occurs, it will not be possible to sterilize the agarose in this manner. If this case, reheat the solution and reattempt filter sterilization. Maintain the sterilized agarose at ~39 °C ± 2 °C for encapsulation of cells.

Papain solution for digestion of matrix—Prepare a 125 µg/ml papain, 0.1 M sodium phosphate (NaH₂PO₄·H₂O), 5 mM EDTA (C₁₀H₁₆N₂O₈), and 5 mM cysteine hydrochloride (HSCH₂CH(NH₂)COOH·HCl) in dH₂O. Note: EDTA requires a pH>8.0 to go into solution. Therefore increase the pH to 8.0 to dissolve all components and then titrate the solution to pH of 6.5 with HCl prior to use.

Sample fixative for histology/immunohistochemistry—Prepare a 4% paraformaldehyde, 100 mM sodium cacodylate into a 50 ml conical tube. If alginate is being fixed, add 50 mM BaCl₂ to irreversibly cross-link the alginate matrix. Titrate the solution with 1 N NaOH to a pH of 7.4. 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.

3. Methods

Overview and time estimates for each method.

1. Pellet culture of ASCs (2 hours to set up on day of experiment).
2. Alginate culture of ASCs (4 hours to set up on day of experiment).
3. Agarose culture of ASCs (4 hours to set up on day of experiment).
4. Histologic and immunohistochemical methods (1 hour to fix samples overnight, 1 day to embed samples, 15 min/sample for sectioning, several days for staining).

Detailed Methods

Cell Culture

- 1 Pellet Culture
 - A. 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.
 - B. Resuspend cells in either Incomplete (control) or Complete Chondrogenic Medium at a density of 500,000 cells/mL.
 - C. Pipette 0.5 ml of cell suspension into 15 mL polypropylene conical tubes. This yields 250,000 ASCs per tube.
 - D. Centrifuge at $300 \times g$ at 21 °C for 5 minutes to form a pellet at the bottom of the tube.
 - E. Loosen the tops of the conical tubes for gas exchange. Incubate cultures at 37°C and 5% CO₂.
 - F. The following day, the pellets will become rounded in the bottom of the tube (Figure 1a).
 - G. 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 are 2, 4, 6, and 8 weeks.
 - H. At each medium exchange, agitate tube gently to ensure the pellet has not adhered to the wall of the tubes. See Figure 1b for an example of chondrogenically vs. control induced ASC pellets after 8 weeks in culture.
 - I. 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 6).

- J.** Incomplete chondrogenic medium (Table 1) can be used as a negative control. Complete chondrogenic medium (Table 2) with ASCs known to be capable of chondrogenesis may be used as a positive control.

2 Making alginate beads:

- A.** Warm alginate and CaCl_2 to 37°C prior to encapsulation of ASCs.
- B.** 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: if the latter technique is used for mixing, a volume of greater than 700 μl is required to avoid making bubbles. (Figure 2)
- C.** Using a 1 ml pipette, draw 1 ml of solution into tip. Note: if larger beads are desired, the tips of the pipettes may be cut with a scalpel (Figure 3a) prior to pipetting to increase the size of the alginate drops.
- D.** 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. Note: 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. See Figures 3b–3c.
- E.** 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.
- F.** Pipette off the CaCl_2 solution. Note: aspiration can be used, but care must be taken to avoid suction pressure on the alginate beads.
- G.** 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 .
- H.** Replace incomplete medium with complete chondrogenic medium with 1 ml/ well in a 24 well plate. Note: typically 1 ml of medium is used for every 800,000 to 1,000,000 cells. A typical culture in one well of a 24 well plate contains 3 beads (Figure 3c).
- I.** Incubate at 37°C , 5.0% CO_2 . 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 are 2, 4, 6, and 8 weeks.
- J.** 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.

3 Making agarose discs:

- A.** Molten agarose should be maintained at $39^\circ\text{C} \pm 2^\circ\text{C}$ prior to encapsulation of ASCs. A magnetic stirrer with hot plate with feedback control may be used to precisely control the temperature (e.g., Figure 4).
- B.** Resuspend ASCs in the 2% agarose solution (temperature = $\sim 39^\circ\text{C}$) 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 stirrer with hot plate (e.g., same process as used with alginate in Figure 2). Note: if the latter technique is used for mixing, a volume of greater than 700 μ l is required to avoid making bubbles. Work quickly while keeping the tube on the hot plate as much as possible during cell mixing to avoid premature gelation.

- C. Quickly draw the molten agarose/cell suspension into a syringe using a 14 gauge needle or larger and add to ASC pellet (Figure 5a). Dispense agarose into desired mold for gelation using ~18 gauge needle (Figure 5b).
- D. Allow agarose to gel for 10 minutes at room temperature in mold.
- E. The gel (e.g., Figure 5c) may be cut with a sterile biopsy punch if desired (Figures 6a–6c). Typically, we have cut our agarose slabs with 6 mm diameter biopsy punches.
- F. Add 1 ml (or appropriate volume) of complete chondrogenic medium or control medium (i.e., incomplete medium) at 1 ml/ well in a 24 well plate. Note: typically 1 ml of medium is used for every 800,000 to 1,000,000 cells. Figure 7 shows 6 mm diameter ASC seeded agarose discs in a 24 well plate.
- G. Incubate at 37°C, 5.0% CO₂. 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 are 2, 4, 6, and 8 weeks.
- H. 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.

Total dsDNA determination

- 4 Samples are harvested at the appropriate time points and are digested in 1 ml of papain solution (for alginate beads, 3 beads/well = 1 construct) for 15–18 hours at 65°C. If desired, digested samples can be stored at –20°C and then thawed for analysis. Note: If wet weight is desired for normalization, weigh the samples before digestion in papain.
- 5 Determine total DNA per construct using the PicoGreen dsDNA quantitation kit per the instructions from the manufacturer. Total dsDNA can then be used, for example, to normalize for total glycosaminoglycan and collagen content (methods not presented herein).

Histologic and immunohistochemical methods

- 6 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.
- 7 Dehydrate constructs with 30% EtOH for 30 min, 50% EtOH for 30 min, 70% EtOH for 30 min (Note: store long term in 70% if not continuing processing), 80% EtOH for 30 min, 100% EtOH for 30 min, followed by one additional 100% EtOH wash (Note: can store overnight at 100% EtOH).
- 8 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. Note: ensure that constructs are translucent. If not clear, continue processing with xylene washes until constructs become clear to translucent.

- 9 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. Note: 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. 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. Note: avoid incubation with molten paraffin for longer times than are needed for complete infiltration as excessive time at 60° C may denature and obscure detection of epitopes needed for immunohistochemistry. 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.
- 10 Cut sections 6–10 µm in thickness with a microtome and place in a 45–50° C waterbath.
- 11 We recommend placing sections on Superfrost®/Plus Microscope Slides (Fisher Scientific or equivalent) by using slides to remove from waterbath. Allow slides to dry overnight in a 37° C slide warmer. Note: these recommended slides have been surface treated for improved adherence of tissue section to slide during processing.

Safranin-o/fast green

- 12 For safranin-o/fast green staining², deparaffinize sections in xylene 3 times for 3 minutes each.
- 13 Re-hydrate in 100% EtOH 2 × 5 minutes, 95% EtOH 1 × 2 minutes, and 70% EtOH 1 × 2 minutes.
- 14 Wash in tap water for 30 sec.
- 15 Stain in Weigert hematoxylin solution for 8 minutes.
- 16 Wash in tap water for 3 minutes by repeatedly dipping slides.
- 17 Differentiate in Differentiation Solution (e.g., Sigma A3179) for 30 seconds.
- 18 Wash in tap water for 10 minutes by repeatedly dipping slides (until sections turn a blue hue).
- 19 Stain in 0.02 % (w/v) aqueous fast green for 3 minutes.
- 20 Rinse for approximately 10 seconds in 1% acetic acid.
- 21 Dip the slides in tap water briefly and then remove excess water from the slide.
- 22 Stain in 0.1 % (v/v) aqueous safranin-O for up to 5 minutes maximum.
- 23 Rinse in 100 % EtOH to remove extra red staining on the slide.
- 24 Dehydrate in 95% EtOH 2 × 5 minutes, followed by 100% alcohol 2 × 5 minutes.

²Nuclei are stained black or dark blue. Bone, muscles (collagen) are stained green, and cartilage is stained orange or red.

- 25 Clear in xylene 3×2 minutes.
- 26 Mount with appropriate mounting solution (e.g., Permount, Fisher Scientific).

Immunohistochemistry

- 27 For immunohistochemistry, we recommend use of Zymed Histostain[®] Plus Broad Spectrum (Invitrogen) or equivalent.
- 28 Deparaffinize by washing slides 3 times $\times$ 2 minutes/wash in xylene.
- 29 Dip slides in 100% EtOH for 2 washes of 2 minutes each.
- 30 After slide dries, circle section with PAP pen.
- 31 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.
- 32 Quench endogenous peroxidase activity by submerging slides in 1 part 30% H₂O₂: 9 parts methanol for 10 minutes.
- 33 Wash slides in D-PBS: 3 washes of 2 minutes each.
- 34 For antigen retrieval, add sufficient pepsin to completely cover each tissue section.
- 35 Incubate at RT for 5 minutes.
- 36 Wash slides in D-PBS: 3 washes of 2 minutes each. (If not labeling for chondroitin sulfate, skip to step 39).
- 37 If using antibodies for labeling chondroitin sulfate epitopes, apply chondroitinase ABC to each section and incubate at RT for 20 minutes.
- 38 Wash slides in D-PBS: 3 washes of 2 minutes each.
- 39 Block sections with Serum Blocking agent.
- 40 Add enough reagent to completely cover tissue section (2–3 drops).
- 41 Incubate at RT for 30 minutes (ensure sections do not dry).
- 42 Blot excess serum from bottom of inclined slide (do not rinse).
- 43 Dilute primary antibody in non-immune serum or 10% goat serum (Note: use recommended dilutions of manufacturer of antibody).
- 44 Add antibody onto (+) staining sections, and blocking serum onto (–) control sections.
- 45 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.
- 46 Wash slides in D-PBS: 3 washes of 2 minutes each.
- 47 Apply appropriate secondary antibody to cover tissue sections (2 drops).
- 48 Incubate at RT for 10 minutes.
- 49 Wash slides in D-PBS: 3 washes of 2 minutes each.
- 50 Apply enough Enzyme Conjugate to cover tissue sections (2 drops).
- 51 Incubate at RT for 10 minutes.

- 52 Wash slides in D-PBS: 3 washes of 2 minutes each.
- 53 Add enough Substrate-Chromagen (e.g., AEC) mixture to cover tissue (2 drops).
- 54 Incubate at RT for 20 minutes.
- 55 Gently dip sections in dH₂O (leave wet).
- 56 Counterstain (Optional, stains cell nuclei).
- 57 Optional: Add 1 – 2 drops Hematoxylin to counterstain nuclei. If slides are not counterstained, skip to step 61. Note: for tissue engineering studies, hematoxylin can sometimes obscure the intracellular and pericellular detection of matrix proteins and therefore should be used with caution.
- 58 Incubate at RT for 5 minutes.
- 59 Wash with tap H₂O.
- 60 Wash slides in D-PBS for 30s or until slides turn a blue hue.
- 61 Wash slides in dH₂O: 3 washes for 2 minutes each (let slides remain in H₂O until coverslips are placed).
- 62 Mount slides by applying mounting medium to 1 slide at a time and place coverslips. Note: do not allow slides to dry before placing mounting medium.

4. Notes

1. Isolation and expansion conditions for ASCS are extremely important (28). The user should familiarize himself/herself with the various techniques and procedures before proceeding with the chondrogenic differentiation protocols presented herein.
2. Depending on culture conditions, donor, cell passage and serum used, it is not uncommon for a percentage of ASCs to undergo apoptosis or necrosis during culture. It is for this reason that we recommend monitoring the dsDNA content as a function of time throughout the culture period.
3. The user is encouraged to maintain samples at day 0 for dsDNA content in addition to histologic and immunohistologic examination. These samples are valuable reference points for the evaluation of the efficacy of chondrogenic differentiation.
4. Care should be taken when encapsulating cells in agarose as too high of a temperature can result in immediate reduction in cell viability. We closely monitor the temperature of the agarose using a temperature probe in a control centrifuge tube, subjected to identical environmental conditions, and also containing an equal amount of agarose as the experimental, sterile agarose. This ensures knowledge of the precise temperature of the experimental agarose solution at all time points during cell encapsulation. (Figure 4)
5. Ultra-low attachment culture plates are used as ASCs that become detached from the matrix will attach to the bottom of standard tissue culture plastic and begin to proliferate. Within days, these cells will overgrow the surface of the well and become a significant variable in the experiment. Use of ultra-low attachment plates will not allow the cells to attach, and the cells can therefore be removed upon medium exchange. An alternate to using purchased ultra-low attachment plates is to coat the wells of standard tissue culture plastic with a thin layer of sterile agarose prior to the start of the experiment.
6. If the cells do not differentiate well, it may be due to a myriad of factors including donor, age of donor, and the specific lot of serum used. Consider combining lots of

ASCs from different donors to minimize variation and screening additional serum lots to ascertain their effect on chondrogenesis. Using different growth factor conditions may also facilitate chondrogenesis (See Table 2).

7. Fresh Safranin-O should always be used for GAG staining, as old (or expired) solutions will result in reduced or no staining of the negatively charged matrix.
8. Safranin-O should not be used with alginate sections as the negatively charged alginate matrix will be heavily stained by the safranin-O.
9. For immunohistochemistry, the user may consider increasing antibody specificity with an alternate secondary antibody as the secondary antibody supplied in immunohistochemistry kits are often broad spectrum in nature. (e.g., Sigma, B7151 diluted 1:400 in D-PBS with 1% BSA could be used as an alternate antibody).

Final Comments

This protocol primarily focuses on 3D culture systems for ASCs with primary qualitative assessment tools using histology and immunohistochemistry to ascertain the degree of chondrogenesis. Other techniques, even though they are beyond the scope of this current protocol, may be appropriate for evaluating chondrogenesis. These assays include, but are not limited, to the following:

1. Dimethyl-Methylene Blue Assay (DMB) to determine glycosaminoglycan content of the constructs (15).
2. Hydroxyproline assay to determine total collagen content of the constructs (15).
3. Quantitative PCR (qPCR) to evaluate transcription of growth factors and extracellular matrix proteins, which can provide early data on the differentiation profile of the ASCs (25,28,31).
4. Mechanical testing to ascertain the development of functional mechanical properties of the constructs during chondrogenesis (15,32).

Finally, this protocol presents three different 3D culture conditions, which have been used by many groups to successfully differentiate ASCs along a chondrogenic lineage. The reader is reminded, however, that these are simple model systems that have been employed, but many other scaffolds have also been described and used successfully for these same purposes and should be considered when selecting a model culture system for chondrogenesis (31–33).

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Figure 1.

(a) appearance of ASC pellet 24 hours after centrifugation in the bottom of a 15 ml conical tube, (b) Left: chondrogenic and Right: control pellets after 8 weeks of in vitro culture. Note: magnification differ between (a) and (b).

Figure 2.

(a) ASC pellet in alginate before mixing, (b) micro stir bar introduced to alginate, (c) micro stir bar prior to mixing, and (d) micro stir bar during mixing. Note that no bubbles are introduced in 2 ml of solution using this method. Note: as low as ~ 700 μ l can be used with this method without introducing bubbles.

Figure 3.

(a) Preparation of pipette tip for making alginate beads - size of bead can be controlled depending on where the tip is cut, (b) Dropping alginate beads into 102 mM CaCl_2 , and (c) cross-linked alginate beads in a 24 well plate.

Figure 4.

Set up for agarose/ASC construct preparation. Magnetic stirrer with hot plate is configured with temperature feedback control: agarose temperature control solution (black arrow) and sterile agarose to be used for experiment (white arrow).

Figure 5. Preparation of agarose cultures, (a) dispensing 2% agarose using a 14 gauge needle onto ASC pellet, (b) Injecting molten agarose into custom mold, and (c) agarose/ASC gel after 10 minute gelation at room temperature.

Figure 6. (a) punching 6 mm diameter discs with a skin biopsy punch, and (b–c) 6 mm diameter ASC-seeded agarose discs after punching.

Figure 7.
ASC-seeded agarose discs (6 mm diameter) in a 24 well plate in 1 ml of medium/well.

Table 1

Incomplete chondrogenic medium

Component	Concentration
DMEM HG	~89%
Fetal Bovine Serum	10%
ITS +	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	100 nM
Penicillin/Streptomycin	1× (100 µg/ml streptomycin, 100 U/ml penicillin, 250 ng/ml Fungizone)

Table 2Growth Factor Combinations for ASC chondrogenesis^{*}

Options	Growth Factors ¹	Final Conc	References
1	TGF- β 1	10 ng/ml	(14,16,23)
2	BMP-6	500 ng/ml	(25,29)
3	TGF- β 3	10 ng/ml	(30)
	BMP-6	10 ng/ml	

^{*} Additionally, 37.5 to 50 μ g/ml L-ascorbic acid 2-phosphate is added freshly to overcome the instability of L-Ascorbic acid 2-phosphate in culture medium. 1000x L-Ascorbic acid 2-phosphate stock can be made in DMEM HG and stored in frozen aliquots for up to six weeks at -20° C.

¹ Siliconized tips and tubes should be used when working with growth factors. This will minimize binding of the growth factors to the pipettes and tubes and theref