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A protocol to prepare decellularized stem cell matrix for rejuvenation of cell expansion and cartilage regeneration

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Abstract

Traditional *ex vivo* expansion of adult stem cells yields an insufficient quantity of less potent cells. Here we describe the fabrication of decellularized matrix deposited by synovium-derived stem cells. This matrix could serve as a three-dimensional expansion system to rejuvenate cells for proliferation and tissue-specific differentiation potential, which could benefit cartilage regeneration. The decellularized stem cell matrix might be a powerful system for tissue engineering and regeneration.

Keywords

Decellularized stem cell matrix; Cell expansion; Synovium-derived stem cells; Chondrogenesis; Cartilage tissue engineering; Cartilage regeneration

1 Introduction

Injury and degenerative diseases such as osteoarthritis are the main causes of cartilage defects. Cartilage defects rarely heal spontaneously due to their intrinsic avascular nature. Despite the fact that both autologous chondrocyte transplantation and stem cell based therapy are promising approaches for the repair of cartilage defects [1], unstable lineage differentiation and an insufficient cell number after traditional two-dimensional cell culture and expansion are the two major challenges for cartilage engineering and regeneration [2].

Physiologically, stem cells reside in the so-called "niche" structure *in vivo*, which is mainly composed of extracellular matrix and soluble factors. Thus, the decellularized extracellular matrix not only can mimic the three-dimensional structure of the stem cell niche but it can also retain the biochemical signals that direct the stem cells to undergo lineage differentiation [3]. Synovium-derived stem cells (SDSCs) have been identified as a tissue-specific stem cell for chondrogenesis [4,5]. To reconstruct an optimal microenvironment for *ex vivo* cell expansion to supplement the shortage of cells for cartilage tissue engineering [6], we developed a decellularized stem cell matrix (DSCM) deposited by SDSCs as an

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expansion substrate with rejuvenation capacities. DSCM expanded cells demonstrated significantly enhanced proliferation and chondrogenic capacity in both porcine and human SDSCs *in vitro* [7–10]. Repair of cartilage defects using DSCM pretreated SDSCs also showed better *in vivo* outcomes when compared to plastic flask expanded SDSCs [11]. So far, we and other labs have performed this technique using bone marrow- [12,13] and adipose-derived mesenchymal stem cells (MSCs) [14], chondrocytes [15], and nucleus pulposus cells [16,17] for tissue-specific regeneration purposes. This intriguing technique would help us further understand the biology of stem cells and their niches.

Here we describe the fabrication of the SDSC deposited DSCM with steps indicated below (Fig. 1). After plating on DSCM, SDSCs had their morphology changed as well as cell proliferation and chondrogenic capacity rejuvenated (Fig. 2). While the protocol presented here primarily focuses on the procedures for preparing DSCM and expanding cells, it is important to note that the mechanisms underlying the rejuvenating effect are exciting [18]. Full characterization of DSCM deposited by SDSCs, including analyses of components of DECM by proteomics, has been attempted [19]. More *in vivo* animal models and human studies about its efficiency and immunity issues are needed before it can be applied in clinically.

2 Materials

2.1 Solutions for Matrix Deposition

1. Gelatin: Weigh 0.2 g of gelatin and dissolve in 100 mL of phosphate buffered saline (PBS) while heating to 37°C. Cool and filter through a 0.2 μm filter. Store at 4°C.
2. Glutaraldehyde: 1% (v/v) solution in PBS and filter through a 0.2 μm filter. Store at 4°C.
3. Ethanolamine: 1 M solution in water and filter through a 0.2 μm filter. Store at 4°C.
4. 1× PBS with Ca^{2+} and Mg^{2+}.
5. T175 flasks/plastic container.

2.2 Culturing SDSCs

1. Growth medium: Alpha Minimum Essential Medium Eagle – Alpha Modifications (alpha MEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL Fungizone.
2. 0.5% Trypsin-Ethylenediaminetetraacetic acid (EDTA).
3. 1× PBS without Ca^{2+} and Mg^{2+}.

2.3 DSCM Fabrication

1. Matrix deposition medium: Ascorbic acid should be freshly prepared prior to use as a 1000× stock concentration of 250 mM in growth medium (from Step 2.2.1.)

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in order to yield a final concentration of 250 μM [20]. Weigh 7.24 g of L-Ascorbic acid phosphate magnesium salt and dissolve in 100 mL growth medium. Filter sterilize with a 0.2 μm filter, aliquot, and store at −20°C.

2. Extraction buffer: 0.5% Triton X-100 and 20 mM NH_4OH in PBS. Filter sterilize and store at 4°C.

3. PBS supplement with antibiotics: 1× PBS with Ca^{2+} and Mg^{2+} containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL Fungizone. Store at 4°C.

2.4 Cell Expansion on DSCM and Multi-lineage Differentiation

1. Growth medium.

2. 0.2% collagenase type II: Dissolve in 1× PBS and 50 mM $CaCl_2$. Filter to sterilize and store at −20°C.

3. Cell strainer (70 μm Nylon).

4. Lineage differentiation media: **Chondrogenic medium** consists of high-glucose Dulbecco's modified Eagle's medium (DMEM), 40 mg/mL proline, 0.1 μM dexamethasone, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM L-Ascorbic acid phosphate magnesium salt, and 1× ITS Premix (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 μg/mL selenous acid, 5.35 μg/mL linoleic acid, and 1.25 μg/mL bovine serum albumin) supplemented with 10 ng/mL transforming growth factor β3. **Adipogenic medium** consists of growth medium supplemented with 1 mM dexamethasone, 0.5 mM isobutyl-1-methyxanthine, 200 μM indomethacin, 10 μM insulin, and 1 nM 3,3'5'-triiodo-L-thyronine. **Osteogenic medium** consists of growth medium supplemented with 0.01 μM dexamethasone, 10 mM β-glycerol phosphate, 50 μM L-Ascorbic acid phosphate magnesium salt, and 0.01 μM 1,25-dihydroxyvitamin D3.

3 Methods

3.1 Surface Preparation for DSCM Deposition

1. To prepare surface for matrix deposition, add enough 0.2% gelatin solution to cover the bottom of a tissue culture plastic flask or dish and incubate at 37°C for 1 h. (scale up the volume of all reagents according to container size)

2. Aspirate gelatin solution and rinse once with PBS for 5 min.

3. Add 1% glutaraldehyde to each flask or dish and incubate at room temperature (RT) for 30 min.

4. Remove glutaraldehyde and wash three times with PBS for 5 min each.

5. Incubate flask or dish with 1 M ethanolamine at RT for 30 min and aspirate ethanolamine.

6. Repeat PBS wash three times for 5 min each.

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7. Aspirate PBS and add growth medium to the flask or dish. If medium turns purple, repeat Steps 3.1.6 and 3.1.7 to remove any trace amounts of ethanolamine.

8. At this point, the surface is prepared and ready to seed cells. Another option is to keep the flask or dish with coated surface in PBS at RT for days (less than one week) under sterile conditions.

3.2 Culturing SDSCs

1. Human SDSCs (primary cells) can be isolated from synovial tissue or obtained from companies. The isolated synovial cells need to be characterized for multi-lineage differentiation capacity as described previously [9] before use in the following experiments.

2. The characterized SDSCs are cultured in growth medium until 95% confluence. Replace growth medium every two days.

3. Rinse the cells with pre-warmed PBS without Ca^{2+} and Mg^{2+} briefly to remove any traces of serum that may inhibit trypsin activity.

4. Add warm 0.5% Trypsin/EDTA to cover the cell layer and let sit for 1 min. Observe under an inverted microscope at RT until the cells shrink and become spherical (about 2 min). Slap sides of the flask or dish to detach cells. Trypsin exposure to the cells should not exceed 4 min.

5. Neutralize the Trypsin/EDTA with proportional growth medium (two-fold volume of Trypsin/EDTA) and collect the cell suspension into centrifuge tube.

6. Centrifuge the cell suspension at 1200 rpm at 4°C for 7 min and discard the supernatant carefully without disturbing the cell pellet.

7. Resuspend the cell pellet with fresh growth medium and count the cells with a hemocytometer.

3.3 DSCM Fabrication Using SDSCs

1. Dilute detached SDSCs to appropriate concentration from Step 3.2 and seed them into gelatin-coated surfaces (from Step 3.1) at 6,000 cells per cm^2 (according to the surface area of the cell containers).

2. Culture SDSCs with growth medium until 100% confluence.

3. Switch to matrix deposition medium (growth medium containing 250 μM L-Ascorbic acid phosphate magnesium salt) and continue culture for another 10 days with medium change every two days [20].

4. Aspirate the medium and rinse flasks carefully with PBS without disturbing the cell layer with matrix.

5. Gently add pre-warmed extraction buffer (*see* Step 2.3) with the matrix surface covered.

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6. Immediately observe cell lysis under an inverted microscope until no intact cells are visualized (about 5 min).
7. Slowly add PBS (same volume as extraction buffer) to dilute cell debris with extraction buffer; avoid touching the matrix with pipet. Gently move the flasks or dishes to 4°C refrigerator and leave them to incubate overnight.
8. Carefully aspirate the diluted cell debris and rinse the matrix with PBS three times.
9. To store the matrix, cover the flasks or dishes with PBS supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL Fungizone. Seal with Parafilm and store at 4°C for up to 4 weeks. (*see* Note 4.1)

3.4 Cell Seeding and Harvesting after DSCM Expansion and Preparation of Multi-lineage Differentiation

1. Warm the DSCM and growth medium to RT.
2. Obtain the cell suspension as described in Step 3.2 and count cell number.
3. Seed SDSCs at about 3000 cells per cm^2 into flask or dish coated with matrix (prepared in Step 3.3) or plastic flasks as a control. Monitor the cell morphology and status of confluence under an inverted microscope. Replace growth medium every two days.
4. After about 8-day expansion or when the cells in the plastic flask reach 95% confluence, aspirate medium and rinse with PBS.
5. Incubate the cells with pre-warmed 0.2% collagenase type II for 20 min until the matrix dissolves.
6. Pipet up and down the mixture of cells and matrices for 2 min until there are no visible pieces of matrix.
7. Filter the cell matrix suspension with 70 μm cell strainer and centrifuge at 1200 rpm at 4°C for 7 min. Discard the supernatant without disturbing the pellet.
8. Resuspend the cell pellet with fresh medium and count cell number with a hemocytometer.
9. Cells are ready to undergo multi-lineage differentiation induction, such as chondrogenic, osteogenic, and adipogenic differentiation.

4 Notes

1. The most critical step of this protocol is to avoid detaching the matrix, especially after decellularization. Special care should be taken when transferring flasks with a large volume of fluids or when the matrix is left to dry. Failing to ensure these attentions can lead to detachment of matrix. The DSCM coated flask scan be stored at 4°C in sterile conditions for up to 4 weeks.

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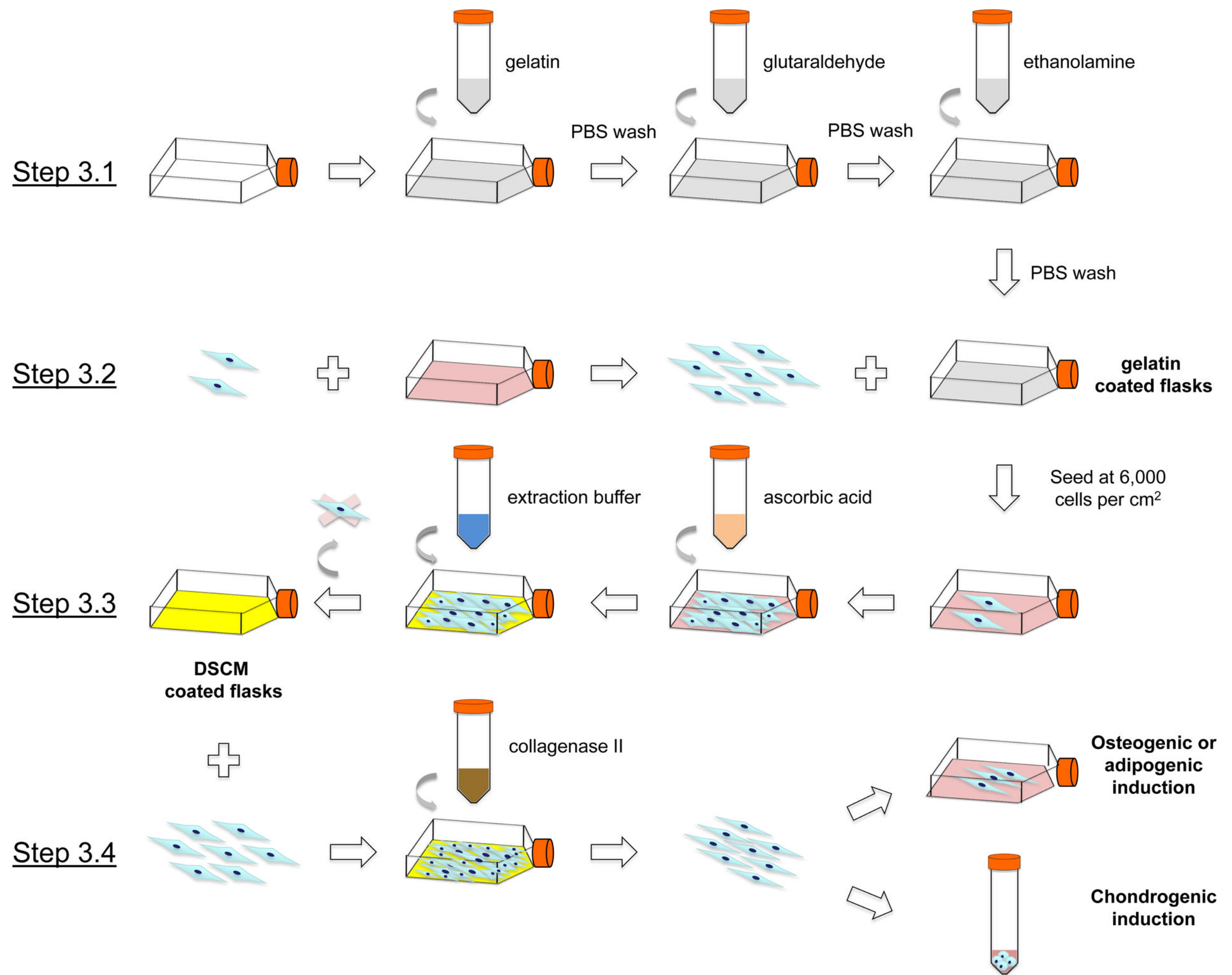

Fig 1.

Step wise preparation of decellularized stem cell matrix (DECM) and synovium-derived stem cell (SDSC) expansion. Step 3.1. Surface Preparation for DSCM Deposition. Step 3.2. Culture of SDSCs. Step 3.3. DSCM Fabrication Using SDSCs. Step 3.4. Cell Seeding and Harvesting after DSCM expansion and Preparation of Multi-lineage Differentiation.

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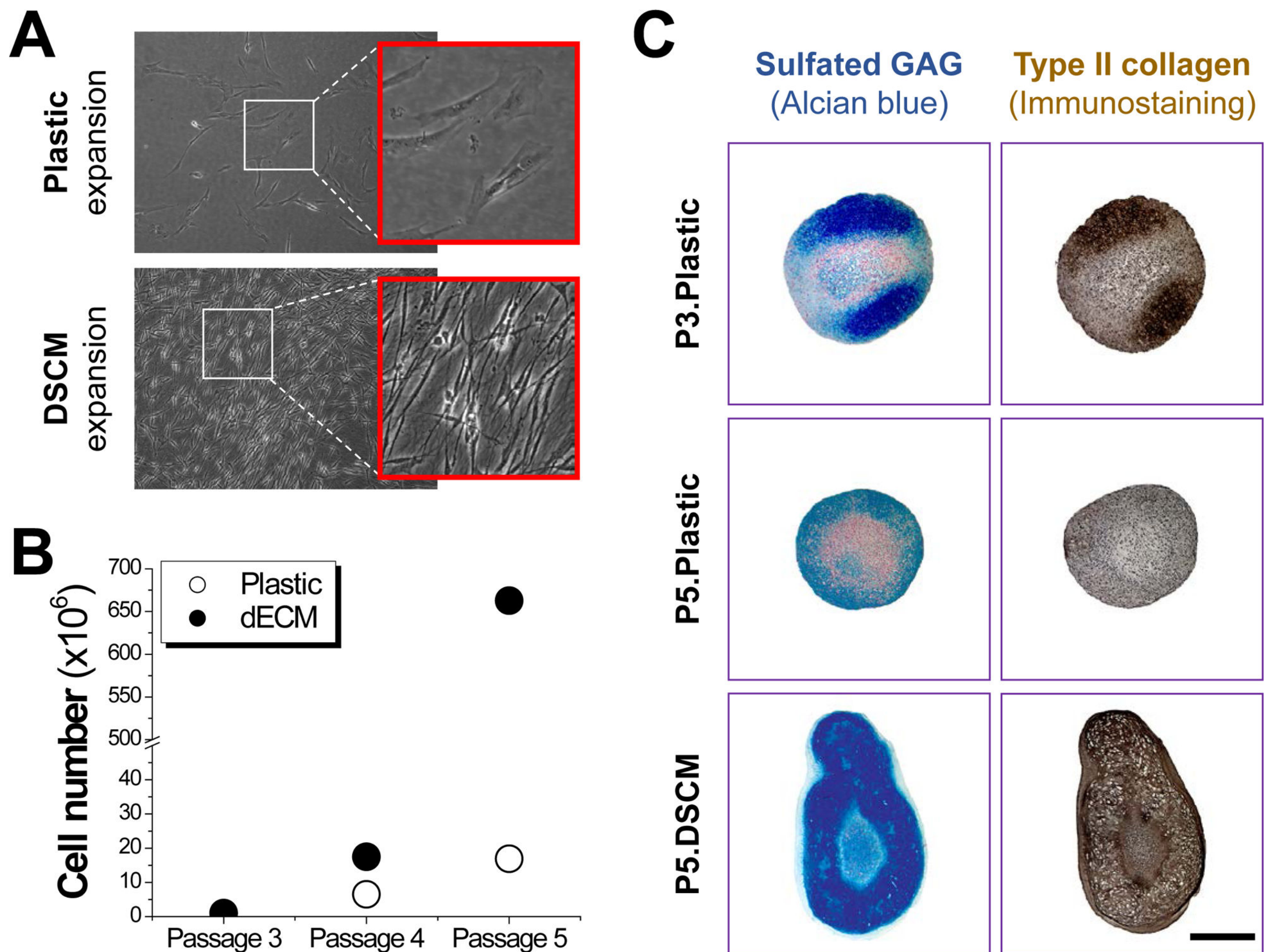

Fig 2.

Effect of decellularized stem cell matrix (DSCM) deposited by synovium-derived stem cells (SDSCs) on porcine SDSCs' proliferation and chondrogenic differentiation. (A) Cell morphology five days after expansion on DSCM and Plastic flasks. (B) Cell proliferation from passage 3 (P3) SDSCs grown on either DSCM or Plastic flasks for two consecutive passages. (C) Alcian blue staining for sulfated glycosaminoglycan (GAG) and immunostaining for type II collagen (scale bar: 800 mm) of two-week chondrogenically induced SDSCs in a pellet culture system after two passages on DSCM (P5.DSCM) or Plastic flasks (P5.Plastic) with pre-expansion SDSCs (P3.Plastic) as a control. Reproduced from ref. 7 with permission from Mary Ann Liebert, Inc. Publications.