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Three-Dimensional Cell Expansion Substrate for Cartilage Tissue Engineering and Regeneration: A Comparison in Decellularized Matrix Deposited by Synovium-Derived Stem Cells and Chondrocytes

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Abstract

Objectives: Synovium-derived stem cells (SDSCs) are tissue-specific stem cells for chondrogenesis. Our aim was to evaluate whether decellularized matrix deposited by SDSCs was superior to chondrocytes in providing a stem cell microenvironment to conduct large scale expansion of high-quality cells for cartilage tissue engineering.

Materials and Methods: We generated two extracellular matrices (ECMs) deposited by either SDSCs (SECM) or chondrocytes (CECM). Passage 4 SDSCs and chondrocytes were expanded separately for two passages on three substrates: conventional plastic flasks (Plastic), SECM, or CECM. Expanded cells were incubated in a pellet culture system supplemented with serum-free chondrogenic medium for 14 days. Histology, biochemistry, real-time PCR, and western blot were used to evaluate expanded cell chondrogenic capacity.

Results: Cell proliferation was greatly improved during expansion on both ECMs, especially on SECM. ECM expansion enhanced cell chondrogenic potential, particularly for cells expanded on SECM. Collagen II and aggrecan were deposited only in CECM while collagen I and decorin existed in both ECMs. High levels of phospho-TGF-β receptor II found in chondrogenically induced cells after expansion on either ECM suggested that enhancement of chondrogenic potential might result from upregulated sensitivity in ECM-expanded cells when they are chondrogenically induced.

Conclusions: SECM is superior to CECM in promoting cell expansion and enhancing expanded cell chondrogenic potential. Decellularized stem cell matrix can serve as a novel cell expansion system for cartilage tissue engineering.

Keywords: Synovium-derived stem cell; Chondrocyte; Decellularized matrix; Cell expansion; Chondrogenesis

Introduction

Articular cartilage is a unique tissue with an avascular structure. Due to a limited self-repair mechanism, cartilage is vulnerable to degenerative disease after trauma or osteoarthritis [1,2]. Autologous chondrocyte transplantation (ACT) has been successfully used in clinical practice to repair cartilage defects [3]. Adult mesenchymal stem cells (MSCs) provide another promising approach for cartilage regeneration. However, the limited proliferative ability and concomitant dedifferentiation during in vitro two-dimensional (2D) expansion hinder chondrocyte and stem cell-based cartilage tissue engineering and regeneration [4,5]. Synovium-derived stem cells (SDSCs) are tissue-specific stem cells for chondrogenesis [6-9]; in contrast, bone marrow stromal cells (BMSCs) are prone to endochondral ossification [10-13]. Our recent work demonstrated that a three-dimensional (3D) extracellular matrix (ECM) deposited by SDSCs provided an in vitro stem cell microenvironment for SDSC expansion, which not only dramatically improved seeded cell proliferation but also enhanced expanded cell chondrogenic potential [14,15].

In the current study, we wondered whether ECM deposited by SDSCs (SECM) could serve as a robust cell expansion system for chondrocyte proliferation and redifferentiation. We were interested to know whether ECM deposited by chondrocytes (CECM) could expand SDSCs and chondrocytes in the same manner that SECM did. We further wanted to determine whether SECM was superior to CECM in enhancing SDSC and chondrocyte proliferation and chondrogenic potential. In this study, we hypothesized that SECM could provide a stem cell microenvironment favoring cell expansion and increasing chondrogenic potential compared to CECM. This study provides more information to the current knowledge in cell-based cartilage tissue engineering and regeneration.

Materials and Methods

Isolation and culture of SDSCs and chondrocytes

This project was approved by the Institutional Animal Care and Use Committee and conducted in compliance with the National Advisory Committee for Laboratory Animal Research Guidelines. Synovium and cartilage tissue were collected aseptically from the knees of two 5-month-old Gottingen minipigs (Marshall Bioresources, North Rose, NY); the synovial tissue was pooled as was the cartilage tissue. Finely minced cartilage was digested with 0.2% collagenase P (Roche, Indianapolis, IN) at 37°C overnight to release chondrocytes. Synovial

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membrane was digested at 37°C for 30 min in 0.1% trypsin (Roche) and then for 2 h in 0.1% collagenase P to release SDSCs. Chondrocytes and SDSCs were plated in expansion medium [α-minimum essential medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL fungizone] at 37°C in a 5% CO_2, 21% O_2 incubator. SDSCs were purified and characterized from primary culture according to our previous report [16].

Preparation of decellularized SECM and CECM

The procedure of obtaining cell-free ECM was described previously [14]. Briefly, passage 3 SDSCs and chondrocytes were seeded on plastic flasks. After reaching 90% confluence, the culture medium for SDSCs was supplemented with 50 µM of ascorbic acid-2-phosphate (Wako, Richmond, VA); in contrast, chondrocytes were incubated in complete medium [high-glucose DMEM, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM HEPES, 0.1 mM non-essential amino acids, 0.4 mM proline, and 50 mg/L L-ascorbic acid (Sigma, St. Louis, MO)]. After 8-day incubation, decellularized ECM was obtained by incubating with 0.5% Triton X-100 (Sigma) containing 20 mM ammonium hydroxide at 37°C for 5 min and stored in phosphate buffered saline at 4°C.

In vitro expansion of SDSCs and chondrocytes

SDSCs and chondrocytes were expanded from passage 4 to passage 6 on three substrates: plastic flasks (Plastic), flasks coated with SECM (SECM), and flasks coated with CECM (CECM). The culture period in each passage was seven days and the expansion medium was changed every two to three days. Cell number was counted using a hemacytometer (Hausser Scientific, Horsham, PA).

Chondrogenic induction of SDSCs and chondrocytes

3×10^5 of expanded SDSCs and chondrocytes from passage 6 were centrifuged to form pellets in 15-mL polypropylene tubes at 500 g for 5 min. After a 24-hour-incubation, the pellets (Day 0) were cultured in a serum-free chondrogenic medium [high-glucose DMEM, 40 µg/mL proline, 100 nM dexamethasone (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM ascorbic acid-2-phosphate and 1 × ITS™ Premix (BD Biosciences, San Diego, CA) with supplementation of 10 ng/mL of TGF-β1 (PeproTech Inc., Rocky Hill, NJ)]. The pellets were collected at Days 0, 7, and 14 for further analysis.

Histology and immunohistochemistry

Samples were fixed in 4% paraformaldehyde overnight, embedded in paraffin blocks, and cut into 5-µm thickness. Sections were stained with Alcian blue and Safranin O to detect sulfated glycosaminoglycans (GAGs). For immunostaining, sections were incubated with primary antibodies against collagen II [II-II6B3, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA], collagen I (Sigma), collagen X (Sigma), and decorin (DSHB), followed by the secondary antibody of biotinylated horse anti-mouse IgG (Vector, Burlingame, CA) and detected by using Vectastain ABC reagent (Vector) with 3,3'-diaminobenzidine (DAB) as a substrate.

Biochemical analysis of DNA and GAG amount

The harvested pellets (n = 4) were digested at 60°C for 6 h with 125 µg/mL papain in PBE buffer (100 mM phosphate, 10 mM EDTA, pH 6.5) containing 10 mmol/L cysteine. The amount of DNA was measured using the Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen) with a CytoFluor® Series 4000 (Applied Biosystems, Foster City, CA). GAG amount was measured using dimethylmethylene blue (DMMB) dye and a Spectronic™ BioMate™ 3 Spectrophotometer (Thermo Scientific, Milford, MA) with bovine chondroitin sulfate (Sigma) as the standard.

Real-time polymerase chain reaction (Real-time PCR)

Total RNA was extracted from pellets (n=4) using TRIzol® (Invitrogen). 1 µg of mRNA was used for reverse transcriptase (RT) with a High-Capacity cDNA Archive Kit at 37°C for 120 min as recommended by the manufacturer (Applied Biosystems). Chondrogenic marker genes (Sox9, collagen II, and aggrecan) and hypertrophic marker gene (collagen X) were customized by Applied Biosystems as part of the Custom Taqman® Gene Expression Assays Table 1. Eukaryotic 18S RNA (Assay ID: HS99999901_s1 ABI) was carried out as the endogenous control gene. Real-time PCR was performed with the iCycler iQ™ Multi Color RT-PCR Detection and calculated by computer software (Perkin-Elmer, Waltham, MA). Relative transcript levels were calculated as $\chi=2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct=\Delta E-\Delta C$, $\Delta E=Ct_{exp}-Ct_{18s}$, and $\Delta C=Ct_{ct1}-Ct_{18s}$.

Western blot

The cytoplasm protein in pellets was extracted using RIPA lysis buffer supplemented with halt protease and phosphatase inhibitor cocktail (Thermo Scientific). The samples were denatured and separated by NuPAGE® Novex® Bis-Tris Mini Gels (Invitrogen) at 200 V for 45 min at 4°C, then transferred onto a nitrocellulose membrane (Invitrogen) using XCell II™ blot module (Invitrogen) at 30V for 1 h at 4°C. The membranes were first blocked in SuperBlock Blocking Buffer (Thermo Scientific), then incubated with primary monoclonal antibodies of phospho-TGF-β receptor (R) II (Tyr 424) (Santa Cruz Biotechnology, Santa Cruz, CA), Sox9 (Abcam, Cambridge, MA), collagen II (II-II6B3, DSHB), and β-actin (Abcam) at the recommended dilution at 4°C overnight. The membranes were incubated in the secondary antibody of goat anti-mouse IgG (H+L) or goat anti-rabbit IgG (H+L) (Thermo Scientific) at room temperature for 40 min followed by SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) and CL-XPosure™ Film (Thermo Scientific).

Statistical analysis

The Kruskal-Wallis test was used for significant differences among all groups and the Mann-Whitney U test was for pairwise comparison. All statistical analyses were performed with SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). p values less than 0.05 were considered statistically significant.

Results

To determine cartilage matrix-associated chemical composition

Name	Primer/Probe	Sequence (5'-3')	Genebank Accession
Aggrecan	Forward	GCCACTGTTACCGCCACTT	X60107
	Reverse	CACTGGCTCTCTGCATCCA	
	Probe	CTGACCGGGCGACCTG	
Col II α1	Forward	TCCTGGCCTCGTGGGT	AF201724
	Reverse	GGGATCCGGGAGAGCCA	
	Probe	CTCCCCTGGGAAACC	
Col X α1	Forward	GGCCCGGCAGGTCATC	NM_001005153
	Reverse	TGGGATGCCTTTTGGTCCTT	
	Probe	TCAGACCTGGTTCCCC	
Sox9	Forward	TGGCAAGGCTGACCTGAAG	AF029696
	Reverse	GCTCAGCTCGCCGATGT	
	Probe	CCCCATCGACTTCCGC	

Table 1: TaqMan customized porcine marker gene primers and probes.

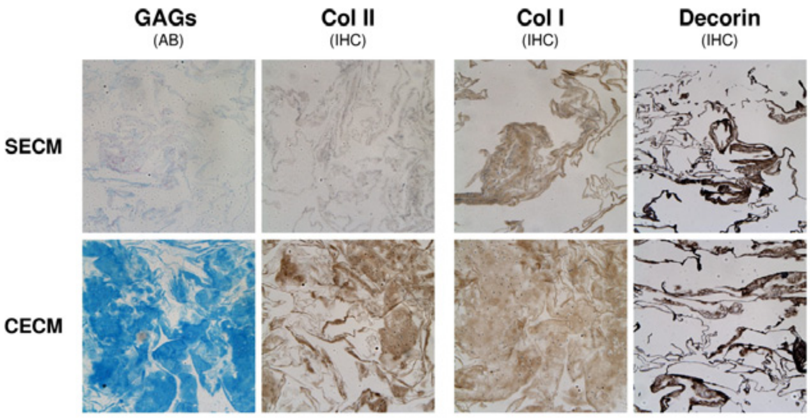

Figure 1: Histology and immunohistochemistry of SECM and CECM. Alcian blue staining (AB, blue color) was used to detect sulfated GAGs and immunohistochemistry staining (IHC, brown color) was for collagens I and II, and decorin.

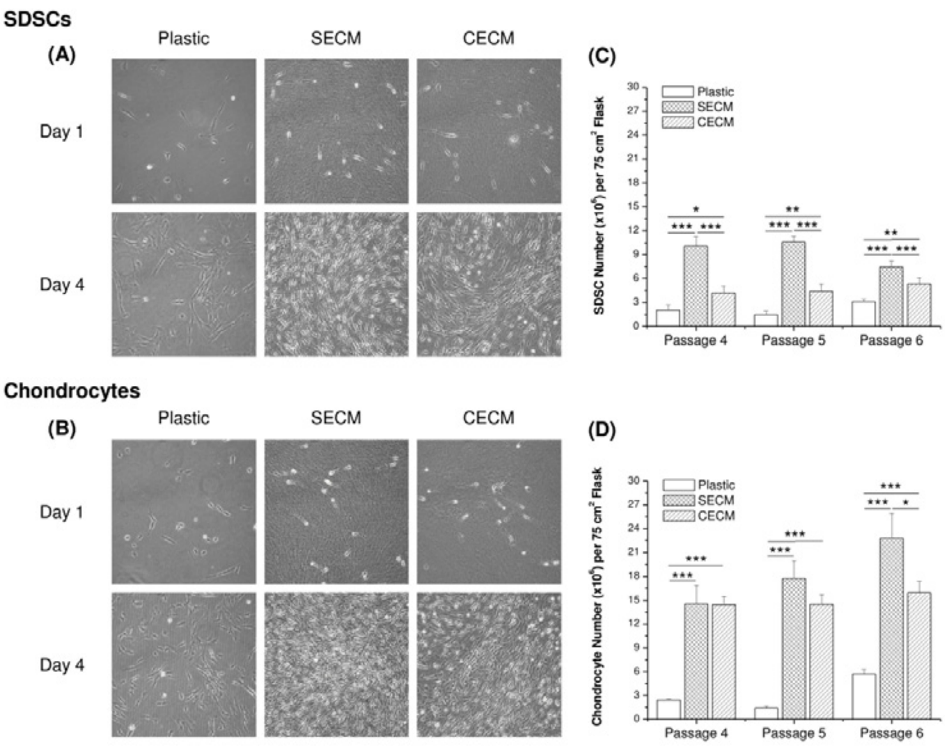

Figure 2: SECM and CECM induces expanded SDSC (A) and chondrocyte (B) morphology change at days 1 and 4; expanded cell number of SDSCs (C) and chondrocytes (D) from passage 4 to passage 6 was quantitatively compared when seeded on Plastic, SECM, and CECM. Significant differences are indicated as follows: * = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$. Data are shown as average ± SD for n = 4.

in SECM and CECM, both ECMs were stained with Alcian blue for sulfated GAGs and immunostained for collagens I and II, and decorin Figure 1. We found that both ECMs exhibited a high expression of collagen I and decorin. Only CECM was intensely stained for sulfated GAGs and collagen II.

To determine the proliferative effect of in vitro microenvironments on expanded cells, SDSCs were employed to prepare an ECM imitating an adult stem cell microenvironment and chondrocytes were used to prepare an ECM imitating a chondrocyte microenvironment. SDSCs and chondrocytes were expanded individually on SECM and CECM with Plastic as a control. After both cells were expanded on either SECM or CECM, cell morphology was observed with significant changes compared to those on Plastic: "spindle-like shape and glistening morphology and smaller size" versus "large and flat shape" Figure 2A/B. From passage 4 to 6, SDSC expansion on SECM yielded a 2.4- to 7.2-fold increase in cell number compared to Plastic and an average 2.0-fold increase in cell number compared to CECM Figure 2C. Despite the fact that passage 4 chondrocytes on SECM and CECM yielded a comparable cell number about 6-fold that of Plastic, passage 5 and 6 chondrocytes on SECM exhibited 20% and 40% higher proliferative ability, respectively, than that on CECM, and maintained a 4.0- and 12.0-fold increase compared to Plastic Figure 2D. Our data

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suggested that both ECMs favored SDSC and chondrocyte expansion; SECM expansion regained the highest proliferative capacity.

To determine whether ECM expansion could improve cell viability in pellets when incubated in a chondrogenic medium (a harsh environment that can induce cell apoptosis), we normalized DNA amounts at days 7 and 14 by those at day 0. Compared to a continuing decrease in cell number in pellets from Plastic expanded cells, ECM expansion reversed this trend by enhancing cell viability and even proliferation in chondrogenically incubated pellets, particularly for SECM expanded SDSCs (51.79 ± 3.83% at day 7 versus 80.99 ± 5.01% at day 14) and chondrocytes (comparable with CECM expanded chondrocytes at day 14, 110.97 ± 6.04% versus 119.01 ± 7.76%) Figure 3.

To determine if ECM pretreatment could enhance expanded cell chondrogenic potential, a 14-day chondrogenic induction was conducted in a pellet culture system. Despite an initial lower GAG amount, SECM expanded SDSCs yielded pellets with the highest GAG amount compared to those from CECM expansion (more than double) and from Plastic expansion (more than 8.5-fold) after a 14-day chondrogenic induction. Interestingly, both ECM expanded chondrocytes yielded pellets with double the GAG amount compared to those from Plastic expansion at either 7 or 14 days after induction despite there being no significant difference between the two ECM groups. Chondrogenic index (GAG/DNA) was consistent with the trend in GAG amount described above Figure 3. Chondrogenic differentiation was also evaluated using histology and immunostaining. Cells expanded on both ECMs formed pellets with intense staining of sulfated GAGs and collagen II compared to those expanded on Plastic. Compared to SDSCs expanded on SECM yielding pellets with a higher intensity of chondrogenic markers than those on CECM, there was similar intensity and size in the pellets from chondrocytes expanded on SECM and CECM. Our histology data were consistent with the biochemistry data. There was no collagen X detectable in any 14-day pellets Figure 4. Quantitative real-time PCR was used to evaluate chondrogenic marker gene expression. Consistent with our above data, SECM expanded SDSCs yielded pellets with higher mRNAs of Sox9, collagen II, and aggrecan compared to those from CECM expanded SDSCs with the lowest gene expression occurring in Plastic expanded SDSCs Figure 5A. SECM expanded chondrocytes and CECM expanded chondrocytes had comparable mRNAs of Sox9, collagen II, and aggrecan; the lowest gene expression occurred in Plastic expanded chondrocytes Figure 5B.

To determine whether TGF-β RII was involved in the enhanced chondrogenic potential in cells expanded on ECMs, western blot was used to evaluate protein expression Figure 6. During chondrogenic induction, phospho-TGF-β RII expression was upregulated in SDSCs and chondrocytes expanded on either SECM or CECM compared to Plastic. Correspondingly, the expression of Sox9 and collagen II were stronger after 14-day chondrogenic induction in both cells expanded on either ECM compared to Plastic. Interestingly, we could not detect collagen II in Plastic-expanded SDSCs even after 14-day chondrogenic induction, which was consistent with our histology and real-time PCR data. This was possibly due to replicative senescence from Plastic expansion (passage 6) which could be overcome by ECM expansion.

Discussion

There is increasing and promising evidence suggesting that ECM can be used as a scaffold for lineage-specific tissue engineering and regeneration [17]. Collagen II hydrogel can enhance chondrogenesis in

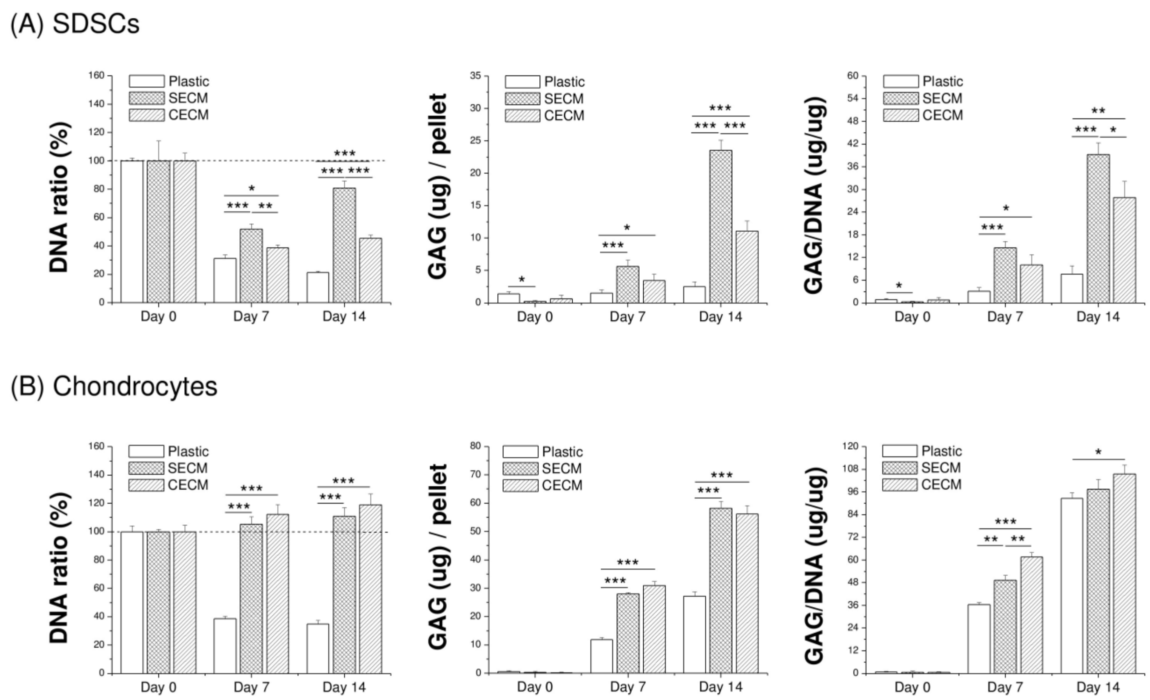

Figure 3: Biochemical analyses were used to detect DNA and GAG amounts in the pellets from expanded SDSCs (A) and chondrocytes (B) after a 14-day serum-free chondrogenic induction. Cell viability was shown as DNA ratio, which was from DNA amount at days 7 and 14 normalized by that at day 0. Cell differentiation was shown as chondrogenic index (ratio of GAG to DNA). Significant differences are indicated as follows: * = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$. Data are shown as average ± SD for n = 4.

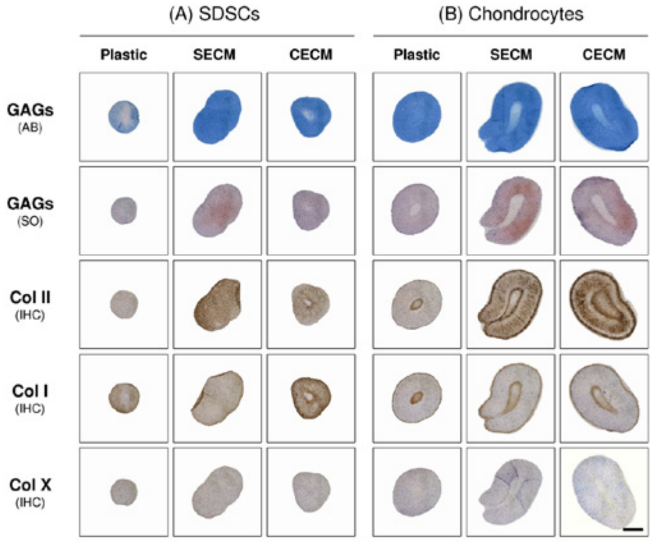

Figure 4: Histology was used to evaluate chondrogenesis of expanded SDSCs (A) and chondrocytes (B) in a pellet culture system after a 14-day serum-free chondrogenic induction. Alcian blue staining (AB, blue color) and Safranin O staining (SO, red color) were used to detect sulfated GAGs, and immunohistochemistry staining (IHC, brown color) was for collagens I, II, and X. The scale bar is 800 μm.

bovine BMSCs [18] and human adipose stem cells (ASCs) [19]. Collagen I hydrogel can provide a 3D microenvironment to retain human chondrocyte phenotypes during proliferation culture [20]. In addition, there are reports demonstrating that porcine ECM, from deposition of either articular chondrocytes or native articular cartilage, can enhance chondrogenic differentiation of rabbit BMSCs [21], human BMSCs [22], and human ASCs [23]. The aim of this study was to determine whether SECM (a tissue-specific stem cell microenvironment) was superior to CECM (a chondrocyte microenvironment) in enhancing SDSC and chondrocyte expansion and chondrogenic potential. Our goal was to find a novel cell expansion system in which candidate cells can be generously multiplied while retaining high quality (sensitivity to chondrogenic induction) for cartilage tissue engineering and regeneration. We found that both SECM and CECM expansion exhibited a robust effect on the enhancement of chondrocyte proliferation and redifferentiation capacity. We also found that SDSCs expanded on SECM displayed a significantly improved effect in SDSC proliferation and chondrogenic potential compared to expansion on CECM, despite the fact that both ECMs were superior to those grown on Plastic. Both ECMs contained ample collagen I and decorin which might contribute to the stem cell microenvironment. During chondrogenic induction, ECM expanded cells exhibited an upregulation of activated TGF-β RII and concomitantly enhanced chondrogenic differentiation.

As we know, cells *in vivo* exist in complex microenvironments, where they constantly interact with multiple ECM molecules rather than a single component. In our previous study, collagen I, a major component in SECM [14], was from ECM preparation using ascorbic acid [8]. In the absence of ascorbic acid, human MSCs produce minimal amounts of collagen, leading to an inhibition of proliferation [24]. Mimicking the expression pattern of native cartilage, 3D collagen I hydrogel could upregulate integrins and downregulate cadherins in chondrocytes, providing sufficient cell preparation and reduced chondrocyte dedifferentiation [20]. In the same way, 3D collagen I hydrogel could upregulate integrin and downregulate multi-lineage differentiation markers in human BMSCs thus retaining their elongated shape [25]. In contrast, CECM was primarily composed of cartilage markers (GAGs and collagen II), representing a cartilage environment. In this study, ECMs were prepared from both SDSCs and chondrocytes at passage 3, which were expected to deposit collagen I in their ECM, particularly for chondrocytes. The presence of collagen I in CECM could be a key factor responsible for the proliferation-promoting effect on seeded cells.

We found both ECMs also contained decorin. Decorin, often found in collagen I-rich matrices, is considered a TGF-β antagonist because it masks the binding site of TGF-β receptors [26,27]. Chondrocytes and SDSCs were detected with a highly phosphorylated level of TGF-β RII during monolayer expansion (on Plastic), which possibly resulted from 2D culture-induced autophosphorylation; spontaneous differentiation caused cells to lose their capacity to respond to the later growth factor-induced lineage-specific differentiation [28,29]. In this

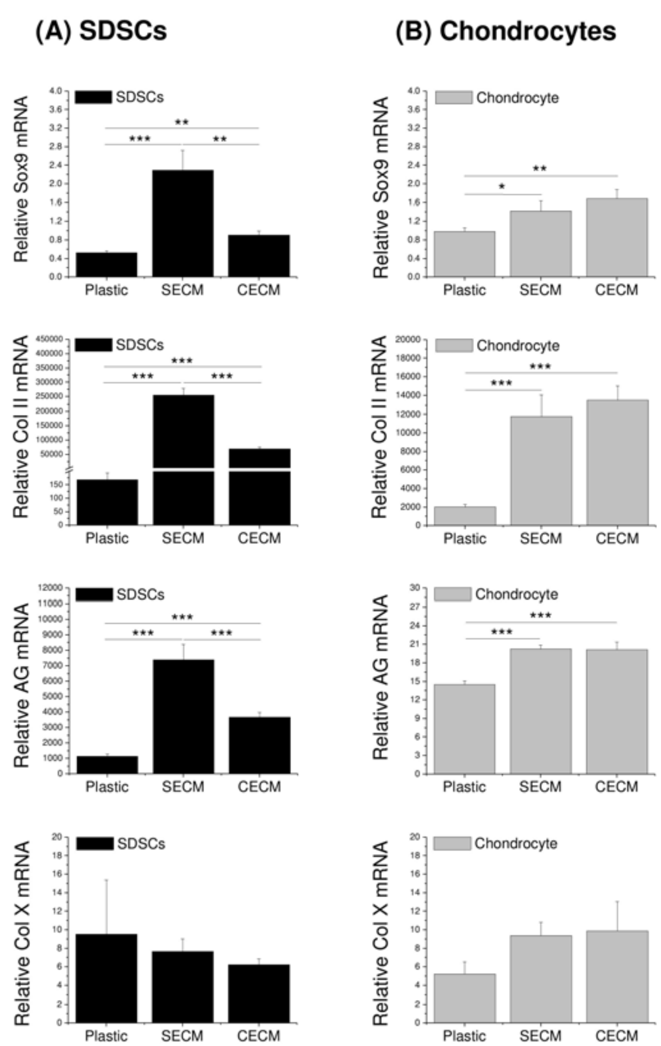

Figure 5: Real-time PCR was used to evaluate mRNA level of chondrogenic marker genes [Sox9, collagen II (Col II), and aggrecan (AG)] and hypertrophic marker gene [collagen X (Col X)] in the pellets from SDSCs (A) and chondrocytes (B) after a 14-day serum-free chondrogenic induction. All 18S RNA normalized genes were shown as a ratio to the value of day 0 pellet cells from Plastic expansion. Significant differences are indicated as follows: * = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$. Data are shown as average ± SD for n = 4.

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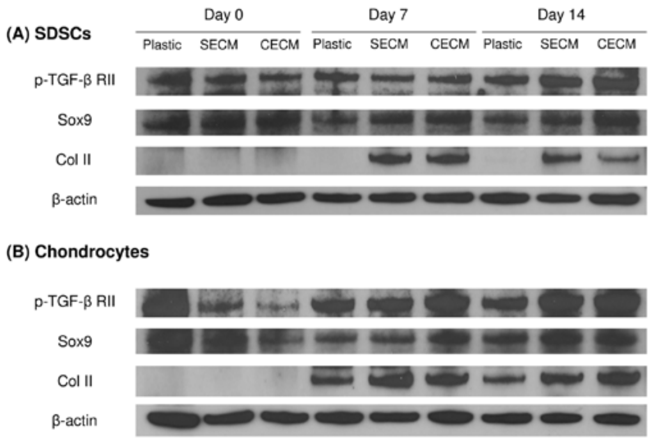

Figure 6: Western blot was used to evaluate protein level of chondrogenic markers (Sox9 and collagen II) and phospho-TGF-β RII (Tyr 424) in the 14-day chondrogenically induced pellets from expanded passage 6 SDSCs and chondrocytes. β-actin was used as an internal control for protein loading.

study, the expression of decorin in ECMs might contribute to the lower level of autophosphorylation of the TGF-β receptor in ECM expanded cells. Upregulation of phosphor-TGF-β RII during chondrogenic induction might cause the enhanced chondrogenic differentiation in ECM expanded cells, in accordance with our previous finding [30]. This study indicated that ECM expansion significantly enhanced the effectiveness of TGF-β induced chondrogenesis possibly by reducing cell autophosphorylation and increasing cell sensitivity to TGF-β.

The application of ECM has inductive properties that favor a specific lineage differentiation [31,32], such as bone matrix favoring human BMSC osteoblast differentiation [33] and cartilage matrix favoring human BMSC chondrogenic differentiation [34,35]. In contrast, in this study, we developed a natural 3D stem cell ECM favoring cell proliferation and chondrogenic potential; comparatively, collagen II-dominated ECM possesses a higher chondrogenic inductive property [19]. Our study indicates that SECM can provide a stem cell microenvironment on which small amounts of SDSCs and chondrocytes can be significantly multiplied with high quality for cartilage regeneration. Since SDSCs and chondrocytes were pooled from two minipigs, both cell expansion on ECM could be considered a substrate from an allogeneic cell source, indicative of the feasibility of expanding SDSCs and chondrocytes from patients on commercially available ECM from young and healthy donors. From the standpoint of translation to the clinics, this approach can be applied in a cost effective and large scale manner.

One limitation of our study was the small number of donor animals. This limitation does not allow us to determine the effect of donor-to-donor variability. Moreover, the underlying mechanism of cell expansion needs to be elucidated before this technique can be transitioned to the clinic.

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Author Disclosure Statement

No competing financial interests exist.

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