Published in final edited form as:

Biomed Mater. ; 11(4): 045009. doi:10.1088/1748-6041/11/4/045009.

Ascorbate-dependent impact on cell-derived matrix in modulation of stiffness and rejuvenation of infrapatellar fat derived stem cells toward chondrogenesis

Tyler Pizzute, B.S.^{1,2,#}, Ying Zhang, Ph.D.^{1,3,#}, Fan He, Ph.D.⁴, and Ming Pei, M.D., Ph.D.^{1,2,3,5}

¹Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, Morgantown, WV, USA

²Exercise Physiology, West Virginia University, Morgantown, WV, USA

³Mechanical and Aerospace Engineering, West Virginia University, Morgantown, WV, USA

⁴Orthopaedic Institute, Medical College, Soochow University, Suzhou, China

⁵Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV, USA

Abstract

Developing an *in vitro* microenvironment using cell-derived decellularized extracellular matrix (dECM) is a promising approach to efficiently expand adult stem cells for cartilage engineering and regeneration. Ascorbic acid serves as a critical stimulus for cells to synthesize collagens, which constitute the major component of dECM. In this study, we hypothesized that optimization of ascorbate treatment would maximize the rejuvenation effect of dECM on expanded stem cells from human infrapatellar fat pad in both proliferation and chondrogenic differentiation. In the duration regimen study, we found that dECM without L-ascorbic acid phosphate (AA) treatment, exhibiting lower stiffness measured by atomic force microscopy, yielded expanded cells with higher proliferation capacity but lower chondrogenic potential when compared to those with varied durations of AA treatment. dECM with 250 μ M of AA treatment for 10 days had better rejuvenation in chondrogenic capacity if the deposited cells were from passage 2 rather than passage 5, despite no significant difference in matrix stiffness. In the dose regimen study, we found that dECMs deposited by varied concentrations of AA yielded expanded cells with higher proliferation capacity despite lower expression levels of stem cell related surface markers. Compared to cells expanded on tissue culture polystyrene, those on dECM exhibited greater chondrogenic potential, particularly for the dECMs with 50 μ M and 250 μ M of AA treatment. With the supplementation of ethyl-3,4-dihydroxybenzoate (EDHB), an inhibitor targeting procollagen synthesis, the dECM with 50 μ M of AA treatment exhibited a dramatic decrease in the rejuvenation effect of expanded cell chondrogenic potential at both mRNA and protein levels despite no significant difference in matrix stiffness. Defined AA treatments during matrix

Corresponding author: Ming Pei MD, PhD, Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, PO Box 9196, One Medical Center Drive, Morgantown, WV 26506-9196, USA, Telephone: 304-293-1072; Fax: 304-293-7070; mpei@hsc.wvu.edu.

[#]Equal contribution to this work.

preparation will benefit dECM-mediated stem cell engineering and future treatments for cartilage defects.

Keywords

Ascorbic acid; Mesenchymal stem cell; Infrapatellar fat pad; Extracellular Matrix; Stiffness; Chondrogenesis

1. Introduction

As a relatively avascular tissue, articular cartilage is particularly susceptible to damaging effects, such as inflammation and premature osteoarthritis (OA), and has a limited capacity for self-restoration following the onset of degenerative disease or acute trauma. Although current orthopedic strategies such as microfracturing, chondral allografting, and autografting have attempted to restore damaged cartilage to its native state, growing evidence supports the incorporation of mesenchymal stem cells (MSCs) into current therapies for the repair of chondral defects [1]. Compared to autologous chondrocytes in aged individuals, MSCs have been shown to be a more promising cell source for cartilage repair [2,3].

MSCs found throughout the body may exhibit similar surface phenotypes and morphology, but sufficient evidence demonstrates that not all MSCs are created equal because they are typically more effective at differentiating along lineages more similar to their native site of harvest [4]. For instance, synovium-derived stem cells (SDSCs) are a tissue-specific stem cell well-suited for chondrogenesis, which show higher chondrogenic potentials, but the least hypertrophy during chondrogenic induction [5]. SDSCs, isolated from a small biopsy of synovial tissue, obtained through minimally invasive arthroscopy [6], have two subtypes: fibrous SDSCs and adipose SDSCs [7]. Different from fibrous SDSCs found in the joint capsule [8], adipose SDSCs from the infrapatellar fat pad (IPFP) (IPSCs) of the patellar tendon might be a better option owing to fewer inflammatory changes in OA IPFP [9].

In vitro expansion is necessary to provide a sufficient cell number for tissue engineering and regeneration, but presents the challenge of replicative senescence [10]. Recently, decellularized extracellular matrix (dECM) deposited by stem cells was found to provide a “niche”-like microenvironment, on which isolated SDSCs could be efficiently expanded without compromised chondrogenic capacity [11–13]. Initial results showing non-detectable expression of HLA-DR [major histocompatibility complex (MHC), class II, DR] in human SDSCs after *ex vivo* expansion on allogeneic dECM [14] demonstrate the feasibility of commercial preparation of these dECM substrates from healthy, young donors [15] for patients in need of autologous transplantation.

Ascorbic acid, also known as vitamin C, is necessary for the synthesis of ECM, particularly for collagen [16, 17]. More evidence indicates that ascorbic acid could not only increase the expression of chondrogenic markers, such as type II collagen and aggrecan, in bovine articular chondrocytes [18] and the mouse embryonic carcinoma-derived cell line ATDC5 [19], but also promote the expression of type X collagen in chicken chondrocytes [20] and

the ATDC5 cell line [19]. Ascorbic acid was also found to stimulate glycosaminoglycan (GAG) synthesis in cultured human skin fibroblasts [21].

Despite the potential of a dECM-mediated strategy as a promising and novel cell expansion system for cartilage engineering and regeneration, the dose and duration regimens of ascorbic acid treatment have not been defined in dECM preparation to maximize its rejuvenation effect on stem cell chondrogenic potential. In this study, we hypothesized that optimization of ascorbate treatment could maximize the rejuvenation effect of dECM on expanded stem cells in both proliferation and chondrogenic differentiation. Due to the importance of matrix elasticity in directing stem cell lineage specification [22], the stiffness of both dECM and expanded cells following the supplementation of ascorbic acid was also characterized.

2. Materials and Methods

2.1. IPSC Culture

Adult human infrapatellar fat pads were harvested from six young patients with acute meniscus or anterior crucial ligament tear (four male and two female, average 22 years old). This study was approved by our Institutional Review Board. Human infrapatellar fat pads were minced and digested in 0.1% trypsin (Roche, Indianapolis, IN) at 37°C for 30 min and then in 0.1% collagenase P (Roche) for 2 h to release cells. IPSCs were collected from the filtrate by centrifugation and plated in a complete medium [alpha minimum essential medium (αMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL fungizone (Invitrogen, Carlsbad, CA)] at 37°C in a humidified 5% CO₂ and 21% O₂ incubator. The medium was changed every three days.

2.2. dECM preparation

The preparation of dECM was described previously [14,23]. Briefly, plastic flasks (PL) were precoated with 0.2% gelatin (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 h and seeded with passage 2 (P2) IPSCs at 6000 cells/cm². After cells reached confluence, L-ascorbic acid phosphate (AA) (Wako Chemicals USA, Inc., Richmond, VA) was added in complete medium to stimulate matrix deposition. After a certain time period (see below design), the deposited dECM was incubated with 0.5% Triton X-100 containing 20 mM ammonium hydroxide at 37°C for 5 min to remove embedded cells and then stored at 4°C in phosphate-buffered saline (PBS) containing 100U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL fungizone.

Two experiments were designed with PL as a control (figure 1) focusing on (1) AA treatment duration regimen (Experiment 1), using 250 µM of AA to treat confluent P2 IPSCs for 0 day (P2E0), 10 days (P2E10), and 20 days (P2E20); an additional group of dECM was prepared using 250 µM of AA to treat confluent P5 IPSCs for 10 days (P5E10); and (2) AA treatment dosage regimen (Experiment 2), using varied concentrations of AA, in terms of 5 µM (E5), 50 µM (E50), 250 µM (E250), and 500 µM (E500), to treat confluent P2 IPSCs for 10 days; an additional group of dECM was prepared using 50 µM of AA combined with 0.4

mM of ethyl-3,4-dihydroxybenzoate (EDHB) (Sigma-Aldrich) (E50EDHB) to treat confluent P2 iPSCs for 10 days.

2.3. Evaluation of expanded cell proliferation index

Passage 3 human iPSCs were seeded for one passage on five substrates (PL, P2E0, P2E10, P2E20, and P5E10) in Experiment 1 and six substrates (PL, E5, E50, E50EDHB, E250, and E500) in Experiment 2. For proliferation index (PI), P3 iPSCs (before expansion) were labeled with CellVue® Claret at 2×10^{-6} M for 5 min according to the manufacturer's protocol (Sigma-Aldrich). After passaging, expanded cells were collected and measured using a BD FACS Calibur™ flow cytometer (dual laser) (BD Biosciences, San Jose, CA). Twenty thousand events of each sample were collected using CellQuest Pro software (BD Biosciences); PI was analyzed by ModFit LT™ version 3.1 (Verity Software House, Topsham, ME).

2.4. Flow cytometry analysis of expanded cell surface phenotypes

The following primary antibodies were used to detect expanded cell surface profiles: CD29 (abcam, Cambridge, MA), CD90 (BD Pharmingen, San Jose, CA), CD105 (BioLegend, San Diego, CA), the stage-specific embryonic antigen 4 (SSEA4) (BioLegend), and isotype-matched IgGs (Beckman Coulter, Fullerton, CA). The secondary antibody was goat anti-mouse IgG (H + L) R-phycoerythrin conjugated (ThermoFisher Scientific, Milford, MA). Samples ($n=3$) of each 2×10^5 expanded cells were incubated on ice in cold PBS containing 0.1% ChromPure Human IgG whole molecule (Jackson ImmunoResearch Laboratories, West Grove, PA) and 1% NaN₃ (Sigma-Aldrich) for 30 min. The cells were then sequentially incubated in the dark in the primary and secondary antibodies for 30 min. Fluorescence was analyzed by a FACS Calibur (BD Biosciences) using FCS Express software package (De Novo Software, Los Angeles, CA).

2.5. Atomic force microscopy (AFM)

Stiffness of culture substrates (dECMs and PL) and corresponding expanded cells was measured using an MFP-3D-BIO AFM (Asylum Research, TE2000-U, Santa Barbara, CA) integrated with an inverted fluorescence microscope (Nikon Eclipse, Ti-U, Nikon Instruments Inc., Melville, NY) and Olympus TR400-PB cantilevers with manufacturer spring constant of 0.09 N/m. For cell elasticity measurement, P3 cells were seeded on Petri dishes with and without dECM coated substrates at 20,000 cells/cm² overnight. The cells were washed with PBS two times and then fixed for 30 min in 4% glutaraldehyde solution (Sigma-Aldrich). The location of the cantilever on the sample was confirmed using a 10× microscopy objective. For quantitative nanomechanical analysis, Sneddon's modification of the Hertz model developed for a four-sided pyramid was employed. The fixed cell sample elasticity (Young's modulus, E) was corrected with the indentation of the tip, δ , through the

following equation: $E = \frac{\pi}{2} \frac{1-\nu^2}{\tan \alpha} \frac{F}{\delta^2}$, where E is the elastic modulus, ν is Poisson's ratio with a value of 0.5 for dECMs and expanded cells, F is the force given by the cantilever deflection multiplied with the cantilever spring constant, α is the open angle used in this study which had a value of 36°, and lastly, δ is the indentation depth [24].

2.6. Chondrogenic induction of expanded cells

After *in vitro* expansion, 0.25×10^6 of iPSCs from each group were centrifuged at 500 g for 5 min in a 15-mL polypropylene tube to form a pellet. After overnight incubation (day 0 sample), the pellets were cultured for 35 days in a serum-free chondrogenic medium consisting of high-glucose Dulbecco's Modified Eagle's Medium (DMEM), 40 $\mu\text{g/mL}$ proline, 100 nM dexamethasone, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 0.1 mM AA, and 1 $\times$ ITS™ Premix [6.25 $\mu\text{g/mL}$ insulin, 6.25 $\mu\text{g/mL}$ transferrin, 6.25 $\mu\text{g/mL}$ selenous acid, 5.35 $\mu\text{g/mL}$ linoleic acid, and 1.25 $\mu\text{g/mL}$ bovine serum albumin (BSA), from BD Biosciences] with supplementation of 10 ng/mL transforming growth factor beta 3 (TGF- β 3, PeproTech Inc., Rocky Hill, NJ). Chondrogenic differentiation was evaluated using histology, immunostaining, biochemical analysis, and TaqMan® real-time polymerase chain reaction (PCR).

Representative pellets (n=3) were fixed in 4% paraformaldehyde at 4°C overnight, followed by dehydrating in a gradient ethanol series, clearing with xylene, and embedding in paraffin blocks. Sections that were 5- μm thick were histochemically stained with Alcian blue (Sigma-Aldrich; counterstained with fast red) for sulfated GAG. For immunohistochemical analysis, the sections were immunolabeled with primary antibodies against type II collagen (II-II6B3; Developmental Studies Hybridoma Bank, Iowa City, IA) followed by the secondary antibody of biotinylated horse anti-mouse IgG (Vector, Burlingame, CA). Immunoactivity was detected using Vectastain ABC reagent (Vector) with 3,3'-diaminobenzidine as a substrate.

Representative pellets (n=4) were digested for 4 h at 60°C with 125 $\mu\text{g/mL}$ papain in PBE buffer (100 mM phosphate and 10 mM ethylenediaminetetraacetic acid, pH 6.5) containing 10 mM cysteine, by using 150 μL enzyme per sample. To quantify cell density, the amount of DNA in the papain digestion was measured using the QuantiT™ PicoGreen® dsDNA assay kit (Invitrogen) with a CytoFluor® Series 4000 (Applied Biosystems, Foster City, CA). GAG was measured using dimethylmethylene blue dye and a Spectronic BioMate 3 Spectrophotometer (ThermoFisher Scientific) with bovine chondroitin sulfate as a standard.

Total RNA was extracted from pellets (n=4) using an RNase-free pestle in TRIzol® (Invitrogen). About 1 μg of mRNA was used for reverse transcription with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) at 37°C for 120 min. Chondrogenic marker related genes [type I collagen (*COL1A1*; assay ID: Hs00164004_m1), type II collagen (*COL2A1*; assay ID: Hs00156568_m1), and hypertrophic marker genes [type X collagen (*COL10A1*; assay ID: H200166657_m1)] were customized by Applied Biosystems as part of the Custom TaqMan® Gene Expression Assays. Eukaryotic 18S RNA (assay ID: Hs99999901_s1) was carried out as the endogenous control gene. Real-time PCR was performed with the iCycler iQ™ Multi-Color Real Time PCR Detection (Perkin-Elmer, Waltham, MA). Relative transcript levels were calculated as $\chi = 2^{-C_t}$, in which $C_t = E - C$, $E = C_{t_{\text{exp}}} - C_{t_{18s}}$, and $C = C_{t_{\text{ctl}}} - C_{t_{18s}}$.

2.7. Statistics

Numerical data are presented as the mean and the standard error of the mean. A Mann-Whitney U test and a linear model with contrast analysis were used for pairwise comparison in biochemistry and real-time PCR data. All statistical analyses were performed with SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). A p value less than 0.05 was considered statistically significant. AFM data analysis was performed by Kruskal Wallis nonparametric tests, with different letters representing the statistical significance ($p < 0.05$) between different groups.

3. Results

3.1. AA treatment duration regimens affected expanded cells' proliferation and elasticity

To determine whether the duration regimens of AA treatment affected proliferation of dECM expanded cells and stiffness of both dECMs and expanded cells, dECMs were prepared in three different conditions: without AA treatment (P2E0), with varied durations of AA treatment (P2E10 and P2E20), and with 10 days' AA treatment of multiple passage cells (P5E10). Flow cytometry data indicated that, when compared to the PL group, both proliferation index (figure 2A/B) and SSEA4 (figure 2C, supplemental figure 1) increased in dECM expanded cells ($n = 10,000$), particularly for the P2E0 group. Interestingly, all dECM expanded cells ($n = 10,000$) exhibited a lower level of stem cell related surface markers (CD29, CD90, and CD105) in both percentage and median fluorescence intensity when compared to PL expanded cells ($n = 10,000$) (figure 2C, supplemental figure 1).

After cell expansion, AFM was used to measure the elasticity of both culture substrates (figure 3A) and corresponding cells (figure 3B) in four chosen groups (PL, P2E0, P2E10, and P5E10). Compared to the elasticity of PL that was considered infinite [25], we found that P2E10 ($n=551$) exhibited a higher Young's modulus than P2E0 ($n=60$) [11.11 ± 11.51 (kPa) versus 3.79 ± 3.31 (kPa)] ($p < 0.05$) indicating that AA treatment for 10 days could significantly increase dECM elasticity, although it did not show statistical significance with P5E10 ($n=212$) [11.11 ± 11.51 (kPa) versus 10.37 ± 9.99 (kPa)] ($p > 0.05$) (figure 3A). Interestingly, the elasticity of expanded iPSCs (figure 3B) was parallel to their corresponding substrate; P2E10 expanded cells ($n=761$) had a higher Young's modulus than the P2E0 ($n=466$) counterpart with PL expanded cells ($n=281$) being the highest [242.59 ± 217.65 (kPa) versus 101.86 ± 106.47 (kPa) versus 80.72 ± 92.98 (kPa)] ($p < 0.05$). Consistent with their corresponding culture substrates, there is no significant difference in Young's modulus of iPSCs after expansion on P2E10 ($n=761$) and P5E10 ($n=842$).

3.2. AA treatment duration regimens affected expanded cells' chondrogenic potential

After a serum-free chondrogenic induction, we found that dECM expanded cells yielded 35-day pellets ($n=3$) with a larger size and more intense staining of sulfated GAG and type II collagen compared to the PL counterpart ($n=3$) (figure 4A). This histology result was in line with quantitative biochemical analysis data (figure 4B), in which dECM expansion significantly increased cell viability (DNA ratio adjusted by that at day 0) in pellets ($n=4$), particularly for the groups of P2E10 and P2E20; cell viability decreased after expansion on dECM deposited by multiple passage cells (P5E10 versus P2E10). Both P2E10 and P2E20

groups also yielded 35-day pellets (n=4) with a higher chondrogenic index (GAG/DNA) compared to the P2E0 and PL counterparts. Real-time PCR data (figure 4C) indicated that both *COL2A1* and *COL10A1* were most upregulated in the pellets (n=4) from the P2E10 and P2E20 groups; in contrast, *COL1A1* was most downregulated in the pellets (n=4) from the P2E10 group and most upregulated in the PL group.

3.3. AA concentrations in dECM preparation affected expanded cells' proliferation and elasticity

Varied concentrations of AA in matrix preparation were evaluated for the influence on proliferation of dECM expanded cells and stiffness of both dECMs and expanded cells. Flow cytometry data (figure 5A/B) showed that expansion on all dECMs yielded cells (n = 10,000) with higher proliferation index and SSEA4 expression level compared to the PL group; both the E50 and E250 groups had the highest proliferation index. Surprisingly, the highest concentration of AA treatment (E500) did not produce a dECM substrate with comparable rejuvenation effect on proliferation and was only marginally greater than E5. The supplementation of EDHB decreased proliferation index of cells expanded from E50 (n = 10,000). All dECM expanded cells (n = 10,000) exhibited a lower level of stem cell related surface markers, including CD29, CD90, and CD105, compared to PL expanded cells (n = 10,000) (figure 5C, supplemental figure 2).

To determine whether dose regimens of AA treatment influenced the stiffness of dECMs and expanded cells, AFM was used to measure the elasticity of both culture substrates (figure 6A) and corresponding cells (figure 6B) in four typical groups (PL, E50, E50EDHB, and E250). AFM data showed that E50 (n=193) exhibited less than half of the Young's modulus compared to E250 (n=228) [0.39 ± 0.37 (kPa) versus 0.88 ± 0.87 (kPa)] ($p < 0.05$), although it did not show statistical significance with EDHB supplementation in the E50EDHB group (n=146) [0.39 ± 0.37 (kPa) versus 0.34 ± 0.35 (kPa)]. Interestingly, the elasticity of expanded iPSCs was parallel to their corresponding substrate; PL expanded cells (n=281) had the highest Young's modulus followed by the E250 counterpart (n=279) with E50 expanded cells (n=272) being the lowest [242.59 ± 217.65 (kPa) versus 74.97 ± 131.54 (kPa) versus 13.57 ± 14.67 (kPa)] ($p < 0.05$). Consistent with their corresponding substrates, there was no significant difference in Young's modulus of iPSCs after expansion on E50 (n=272) and E50EDHB (n=165).

3.4. AA concentrations in dECM preparation affected expanded cells' chondrogenic potential

Histology data (figure 7A) showed that, compared to the PL group, dECM expanded cells yielded 35-day pellets (n=3) with larger size and more intense staining of sulfated GAG and type II collagen. This finding was confirmed by biochemical data (figure 7B), in which E250 expanded cells yielded pellets (n=4) with the highest ratio of GAG to DNA followed by the E50 group; the supplementation of EDHB significantly decreased the ratio of GAG to DNA; similar to proliferation index data, the highest concentration of AA treatment (E500) did not make dECM with a comparable rejuvenation effect on chondrogenic differentiation. Interestingly, real-time PCR data (figure 7C) showed that E250 expanded cells yielded pellets (n=4) with the highest upregulation of *COL2A1* and *COL10A1*, which was

dramatically downregulated by supplementation of EDHB in preparation of dECM. Meanwhile, *COL1A1* was most downregulated in the pellets from the E250 group and most upregulated in the PL group.

4. Discussion

Ascorbate-guided matrix deposition by cells is becoming a promising cell expansion approach for tissue engineering and regeneration [13,23,26,27]; thus, some important parameters in the use of AA, such as dose and duration regimens, are critical for the success of stem cell rejuvenation in both cell proliferation and differentiation phases. In the experiment targeting AA treatment duration regimens, we found that dECM with AA treatment exhibited an increase in matrix stiffness, which might be due to an ascorbate mediated increase of lysine hydroxylase activity and a great decrease in proline hydroxylase [16], which are responsible for synthesizing the unique amino acids hydroxyproline and hydroxylysine, respectively, contributing to the stability and maturation of collagen [28]. dECM without AA treatment promoted higher cell proliferation while dECM with AA treatment for 10 days promoted higher chondrogenic potential of expanded cells, indicating that there might be a correlation between dECM stiffness and expanded cell chondrogenic capacity. Interestingly, dECM with AA treatment for 20 days did not exhibit further enhanced chondrogenic rejuvenation in expanded cells compared to the 10-day-treatment group.

Our previous study demonstrated that dECM deposited by fetal donors provided an advantage over that produced by adult donors in rejuvenating adult stem cell chondrogenesis [15]. In this study, dECMs deposited by the same cell population but different passaging phase were compared in the rejuvenation effect on expanded cells. Consistent with the previous report [15], we found that dECM deposited by “old” iPSCs (P5) had less effect than that produced by relatively “young” cells (P2) in rejuvenating stem cell chondrogenic potential. As observed in human epithelial tissue, it is believed that the loss of tissue elasticity with aging results from increasing rigidity of the ECM, mostly due to an increase of polymerization of collagen and elastin [29], which corroborates a previous report in which dECM from fetal donors containing unique proteins such as collagen and elastin favored adult SDSC rejuvenation [15]. The use of multiple passage cells (P5) for dECM and the subsequent reduced chondrogenic potential in this study may be linked to age associated biomechanical stress and biochemical composition [2], leading to an altered dECM construct.

We noticed that, in measuring the elasticity of expanded cells, the 4% glutaraldehyde solution used in this study to fix expanded cells led to increased cell elasticity values compared to unfixed cells [15]. Despite ongoing controversy, there is increasing evidence suggesting that glutaraldehyde fixation is the preferred method for reliable data analysis (reproducible) of both cell elasticity and cell morphology [23,30–32].

In the experiment targeting AA treatment dose regimens, we found that both 50 and 250 μ M of AA treatments maximized the rejuvenation effect of dECMs on expanded cells, in terms of proliferation and chondrogenic potential. Interestingly, the supplementation of 0.4 mM

EDHB in dECM preparation with 50 μ M of AA treatment dramatically decreased the rejuvenation effect of dECM preparation with 50 μ M of AA treatment on expanded cells, which might be explained by the inhibition effect of EDHB on collagen synthesis. Majamaa and coworkers demonstrated that 0.4 mM EDHB could inhibit the synthesis of 4-hydroxyproline in normal human skin fibroblast cell culture as a result of reduced prolyl4-hydroxylase activity, and the synthesis and secretion of both type I and type III procollagens were markedly reduced [33]. There is no significant difference in the stiffness of dECMs, regardless of EDHB supplementation, indicating that not only collagens, but also other matrix components, might contribute to matrix stiffness [34].

Extracellular matrix represents an advanced network of proteins with form and function, composed of structural proteins, collagens, and proteoglycans which, as a whole, present a local mechanical microenvironment to the surrounding cells. The cells, influenced by their local microenvironment, sense their surroundings through mechanical cues such as matrix elasticity and through other soluble and insoluble factors presented to them by the matrix [35]. As more research is conducted to elucidate the relationship between matrix rigidity and the determination of cell fate [36,37], the importance of mechanical properties on the biological function of cells and tissues will become more apparent [38]. It is also necessary to note that dECM elasticity in itself is not a completely independent factor affecting cell determination, as other variables can influence the properties of ECM in terms of protein anchoring, porosity, and crosslinking density [39]. By understanding and ultimately controlling the mechanical properties involved in cell-matrix interactions, it is possible to design biological substrates, biomaterials, and scaffolds which most accurately mimic the *in vivo* microenvironment and allow for optimal conditions to facilitate stem cell growth and differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Suzanne Danley for editing the manuscript. We also thank Chenbo Dong and Dr. Cerasela Zoica Dinu for their help with the AFM instrument. This project was partially supported by Research Grants from the Musculoskeletal Transplant Foundation (MTF) and the National Institutes of Health (AR062763-01A1). No competing financial interests exist.

References

1. Karnes, J.; Zhang, Y.; Pei, M. Cell Therapy for the Creation of Cartilage and Related Clinical Trials. In: Templeton, NS., editor. *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies*. 4. Taylor & Francis/CRC Press; 2014. p. 1123-35.
2. Lynch K, Pei M. Age associated communication between cells and matrix: a potential impact on stem cell-based tissue regeneration strategies. *Organogenesis*. 2014; 10:289–98. [PubMed: 25482504]
3. Parsch D, Fellenberg J, Brümmendorf TH, Eschlbeck AM, Richter W. Telomere length and telomerase activity during expansion and differentiation of human mesenchymal stem cells and chondrocytes. *J Mol Med*. 2004; 82:49–55. [PubMed: 14647922]
4. Pizzute T, Lynch K, Pei M. Impact of tissue-specific stem cells on lineage specific differentiation: a focus on musculoskeletal system. *Stem Cell Rev Rep*. 2015; 11:119–32.

5. Jones B, Pei M. Synovium-derived stem cells: a tissue-specific stem cell for cartilage tissue engineering and regeneration. *Tissue Eng Part B Rev.* 2012; 18:301–11. [PubMed: 22429320]
6. Li JT, Campbell D, Bal G, Pei M. Can arthroscopically harvested synovial stem cells be preferentially sorted using SSEA4 antibody for mesenchymal tissue regeneration? *Arthroscopy.* 2014; 30:352–61. [PubMed: 24581260]
7. Mochizuki T, Muneta T, Sakaguchi Y, Nimura A, Yokoyama A, Koga H, Sekiya I. Higher chondrogenic potential of fibrous synovium- and adipose synovium-derived cells compared with subcutaneous fat-derived cells: distinguishing properties of mesenchymal stem cells in humans. *Arthritis Rheum.* 2006; 54:843–53. [PubMed: 16508965]
8. Pei M, He F, Vunjak-Novakovic G. Synovium-derived stem cell-based chondrogenesis. *Differentiation.* 2008; 76:1044–56. [PubMed: 18637024]
9. Loeuille D, et al. Macroscopic and microscopic features of synovial membrane inflammation in the osteoarthritic knee: correlating magnetic resonance imaging findings with disease severity. *Arthritis Rheum.* 2005; 52:3492–501. [PubMed: 16255041]
10. Li JT, Pei M. Cell senescence: a challenge in cartilage engineering and regeneration. *Tissue Eng Part B Rev.* 2012; 18:270–87. [PubMed: 22273114]
11. He F, Chen XD, Pei M. Reconstruction of an in vitro tissue-specific microenvironment to rejuvenate synovium-derived stem cells for cartilage tissue engineering. *Tissue Eng Part A.* 2009; 15:3809–21. [PubMed: 19545204]
12. He F, Pei M. Extracellular matrix enhances differentiation of adipose stem cells from infrapatellar fat pad toward chondrogenesis. *J Tissue Eng Regen Med.* 2013; 7:73–84. [PubMed: 22095700]
13. Pei M, Li JT, Shoukry M, Zhang Y. A review of decellularized stem cell matrix: a novel cell expansion system for cartilage tissue engineering. *Eur Cell Mater.* 2011; 22:333–43. [PubMed: 22116651]
14. Zhang Y, Pizzute T, Li J, He F, Pei M. sb203580 preconditioning recharges matrix-expanded human adult stem cells for chondrogenesis in an inflammatory environment - A feasible approach for autologous stem cell based osteoarthritic cartilage repair. *Biomaterials.* 2015; 64:88–97. [PubMed: 26122165]
15. Li JT, Hansen K, Zhang Y, Dong CB, Dinu C, Dzieciatkowska M, Pei M. Rejuvenation of chondrogenic potential by young stem cell microenvironment. *Biomaterials.* 2014; 35:642–53. [PubMed: 24148243]
16. Murad S, Grove D, Lindberg KA, Reynolds G, Sivarajah A, Pinnell SR. Regulation of collagen synthesis by ascorbic acid. *Proc Natl Acad Sci U S A.* 1981; 78:2879–82. [PubMed: 6265920]
17. Wei F, Qu C, Song T, Ding G, Fan Z, Liu D, Liu Y, Zhang C, Shi S, Wang S. Vitamin C treatment promotes mesenchymal stem cell sheet formation and tissue regeneration by elevating telomerase activity. *J Cell Physiol.* 2012; 227:3216–24. [PubMed: 22105792]
18. Hering TM, Kollar J, Huynh TD, Varelas JB, Sandell LJ. Modulation of extracellular matrix gene expression in bovine high-density chondrocyte cultures by ascorbic acid and enzymatic resuspension. *Arch Biochem Biophys.* 1994; 314:90–8. [PubMed: 7944410]
19. Altaf FM, Hering TM, Kazmi NH, Yoo JU, Johnstone B. Ascorbate-enhanced chondrogenesis of ATDC5 cells. *Eur Cells Mater.* 2006; 12:64–70.
20. Farquharson C, Berry JL, Mawer EB, Seawright E, Whitehead CC. Ascorbic acid-induced chondrocyte terminal differentiation: the role of the extracellular matrix and 1,25-dihydroxyvitamin D. *Eur J Cell Biol.* 1998; 76:110–8. [PubMed: 9696350]
21. Kao J, Huey G, Kao R, Stern R. Ascorbic acid stimulates production of glycosaminoglycans in cultured fibroblasts. *Exp Mol Pathol.* 1990; 53:1–10. [PubMed: 2209807]
22. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell.* 2006; 126:677–89. [PubMed: 16923388]
23. Zhang Y, Li J, Davis ME, Pei M. Delineation of in vitro chondrogenesis of human synovial stem cells following preconditioning using decellularized matrix. *Acta Biomater.* 2015; 20:39–50. [PubMed: 25861949]
24. Hassan A-E, Heinz WF, Antonik MD, D'Costa NP, Nageswaran S, Schoenenberger CA, Hoh JH. Relative microelastic mapping of living cells by atomic force microscopy. *Biophys J.* 1998; 74:1564–78. [PubMed: 9512052]

25. Bhana B, Iyer RK, Chen WL, Zhao R, Sider KL, Likhitpanichkul M, Simmons CA, Radisic M. Influence of substrate stiffness on the phenotype of heart cells. *Biotechnol Bioeng*. 2010; 105:1148–60. [PubMed: 20014437]
26. Lin H, Yang G, Tan J, Tuan RS. Influence of decellularized matrix derived from human mesenchymal stem cells on their proliferation, migration and multi-lineage differentiation potential. *Biomaterials*. 2012; 33:4480–9. [PubMed: 22459197]
27. Pei M, He F, Li JT, Tidwell J, Jones A, McDonough EB. Repair of large animal partial-thickness cartilage defects using matrix rejuvenated synovium-derived stem cells. *Tissue Eng Part A*. 2013; 19:1144–54. [PubMed: 23216161]
28. Murad S, Sivarajah A, Pinnell SR. Regulation of prolyl and lysyl hydroxylase activities in cultured human skin fibroblasts by ascorbic acid. *Biochem Biophys Res Commun*. 1981; 101:868–75. [PubMed: 6272771]
29. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A*. 1995; 92:9363–7. [PubMed: 7568133]
30. Targosz-Korecka M, Brzezinka GD, Danilkiewicz J, Rajfur Z, Szymonski M. Glutaraldehyde fixation preserves the trend of elasticity alterations for endothelial cells exposed to TNF- α . *Cytoskeleton (Hoboken)*. 2015; 72:124–30. [PubMed: 25786919]
31. Dong C, Kashon ML, Lowry D, Dordick JS, Reynolds SH, Rojanasakul Y, Sargent LM, Dinu CZ. Exposure to carbon nanotubes leads to changes in the cellular biomechanics. *Adv Healthc Mater*. 2013; 2:945–51. [PubMed: 23335423]
32. Rheinlaender J, Geisse NA, Proksch R, Schäffer TE. Comparison of scanning ion conductance microscopy with atomic force microscopy for cell imaging. *Langmuir*. 2011; 27:697–704. [PubMed: 21158392]
33. Majamaa K, Sasaki T, Uitto J. Inhibition of prolyl hydroxylation during collagen biosynthesis in human skin fibroblast cultures by ethyl 3,4-dihydroxybenzoate. *J Invest Dermatol*. 1987; 89:405–9. [PubMed: 2822818]
34. Soucy PA, Werbin J, Heinz W, Hoh JH, Romer LH. Microelastic properties of lung cell-derived extracellular matrix. *Acta Biomater*. 2011; 7:96–105. [PubMed: 20656080]
35. Wells RG, Discher DE. Matrix elasticity, cytoskeletal tension, and TGF-beta: the insoluble and soluble meet. *Sci Signal*. 2008; 1:pe13. [PubMed: 18334714]
36. Handorf AM, Zhou Y, Halanski MA, Li WJ. Tissue stiffness dictates development, homeostasis, and disease progression. *Organogenesis*. 2015; 11:1–15. [PubMed: 25915734]
37. Zhang Y, Chen S, Pei M. Biomechanical signals guiding stem cell cartilage engineering: from molecular adaption to tissue functionality. *Eur Cell Mater*. 2016; 30:59–78. [PubMed: 26728499]
38. Levental I, Georges PC, Janmey PA. Soft biological materials and their impact on cell function. *Soft Matter*. 2007; 3:299–306.
39. Trappmann B, et al. Extracellular-matrix tethering regulates stem-cell fate. *Nat Mater*. 2012; 11:642–9. [PubMed: 22635042]

Experiment 1: duration regimen of AA treatment study

Experiment 2: dose regimen of AA treatment study

Figure 1.
Study design.

Figure 2.

Expanded cell proliferation and expression of stem cell surface markers in the duration regimens of AA treatment study. dECMs were prepared with either P2 or P5 iPSCs under varied periods (0 day: E0; 10 days: E10; and 20 days: E20) of 250 μ M AA treatment. Passage 3 iPSCs were expanded on PL, P2E0, P2E10, P2E20, and P5E10 for one passage. Flow cytometry was used to measure proliferation index of expanded cells (A/B) and both percentage and median fluorescence intensity of MSC surface markers (CD29, CD90, and CD105) and SSEA4 of expanded cells (C).

Figure 3.

Biomechanical evaluation of both culture substrates and expanded cells in the duration regimens of AA treatment study. AFM was used to measure stiffness of each dECM group (PL, P2E0, P2E10, and P5E10) (average sample size $n=274$) (A) and corresponding IPSCs (average sample size $n=587$) grown on each above substrates (B). The stiffness of PL (A) is considered as infinite [20]. * $p < 0.05$ indicates a statistically significant difference. NS: no statistically significant difference.

Figure 4.

Expanded cell chondrogenic potential in the duration regimens of AA treatment study. Passage 3 iPSCs expanded on PL, P2E0, P2E10, P2E20, and P5E10 for one passage were followed by a 35-day chondrogenic induction in a pellet system. (A) Pellets were photographed before histology; Alcian blue (Ab) was used to stain sulfated GAG and immunohistochemical staining (IHC) was for type II collagen (Col II). (B) Biochemical analysis was used for DNA and GAG amounts in chondrogenic pellets. Cell viability in a pellet culture system was evaluated using DNA ratio (DNA amount at days 14 and 35 adjusted by that at day 0). Chondrogenic index was evaluated using the ratio of GAG to DNA. (C) Quantitative real-time PCR was used to quantify chondrogenesis related genes,

including *COL1A1*, *COL2A1*, and *COL10A1* in chondrogenic pellets. Data are shown as average $\pm$ SD for n=4. * $p<0.05$ indicates a significant difference.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5.

Expanded cell proliferation and expression of stem cell surface markers in the dose regimens of AA treatment study. dECMs were prepared with P2 iPSCs under varied doses of AA treatment (5 μ M: E5; 50 μ M: E50; 50 μ M + 0.4 mM EDHB: E50EDHB; 250 μ M: E250; and 500 μ M: E500). Passage 3 iPSCs were expanded on PL and the above dECMs for one passage. Flow cytometry was used to measure proliferation index of expanded cells (A/B) and both percentage and median fluorescence intensity of MSC surface markers (CD29, CD90, and CD105) and SSEA4 of expanded cells (C).

Figure 6.

Biomechanical evaluation of both culture substrates and expanded cells in the dose regimens of AA treatment study. AFM was used to measure stiffness of each dECM group (PL, P50, E50EDHB, and E250) (average sample size $n=189$) (A) and corresponding iPSCs (average sample size $n=250$) grown on each above substrates (B). The stiffness of PL (A) is considered as infinite.²⁰ * $p < 0.05$ indicates a statistically significant difference. NS: no statistically significant difference.

Figure 7.

Expanded cell chondrogenic potential in the dose regimens of AA treatment study. Passage 3 iPSCs expanded on PL, E5, E50, E50EDHB, E250, and E500 for one passage were followed by a 35-day chondrogenic induction in a pellet system. (A) Pellets were photographed before histology; Alcian blue (Ab) was used to stain sulfated GAG and immunohistochemical staining (IHC) was for type II collagen (Col II). (B) Biochemical analysis was used for DNA and GAG amounts in chondrogenic pellets. Cell viability in a pellet culture system was evaluated using DNA ratio (DNA amount at days 14 and 35 adjusted by that at day 0). Chondrogenic index was evaluated using the ratio of GAG to DNA. (C) Quantitative real-time PCR was used to quantify chondrogenesis related genes, including *COL1A1*, *COL2A1*, and *COL10A1* in chondrogenic pellets. Data are shown as average $\pm$ SD for $n=4$. * $p<0.05$ indicates a significant difference.