Fabrication of Extracellular Matrix-Based Hydrogel System for Meniscus Repair: Donor Age-Dependent Effects on Meniscal Fibrochondrocytes

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Introduction

The meniscus serves an important role in load-bearing and distribution in the knee.[1] Injuries to this tissue can greatly influence joint motion and daily living.[2] Meniscus injuries occur frequently,[3] and their insufficient repair frequently lead to potential osteoarthritis. Since the meniscus tissue unfortunately has limited vascularity and deficient healing capacity, new therapeutic strategies are required for treating meniscal injuries.[4] Recently, various biomaterials have been introduced to develop tissue engineered scaffolds for meniscal repair and replacement. In particular, decellularized meniscus extracellular matrix (Me-DEM), a bio-ink material capable of 3D cell printing, has been introduced as a promising bioactive material for meniscus regeneration given its bioactive properties.[5] In addition, it is known that the ECM components change with tissue development impacting cellular phenotypes and functionalities.[6] However, it is still unclear how changes in ECM components with the meniscus development impacts meniscus cell behaviors and their regenerative capacities. Thus, in this study, we fabricated age-dependent DEM [extracted from fetal meniscus dECM (FDEM) or adult meniscus dECM (ADEM)]-based hydrogel systems, and further we evaluated how the age-dependent DEM regulates gene expression, proliferation, and matrix productions differently in juvenile bovine meniscal fibrochondrocytes.

Methods

Fetal (3rd trimester) and adult (< 30 months) bovine menisci were collected and decellularized in 0.3% SDS/PBS (w/v), 3% Triton-X100/PBS (v/v), and 7.5U/ml Deoxyribonuclease/PBS. FDEM and ADEM pre-gels were prepared by digesting in 0.5M acetic acid solution (Figure 1A).[5,7] Hematoxylin and eosin (H&E) staining was carried out to evaluate the decellularization and

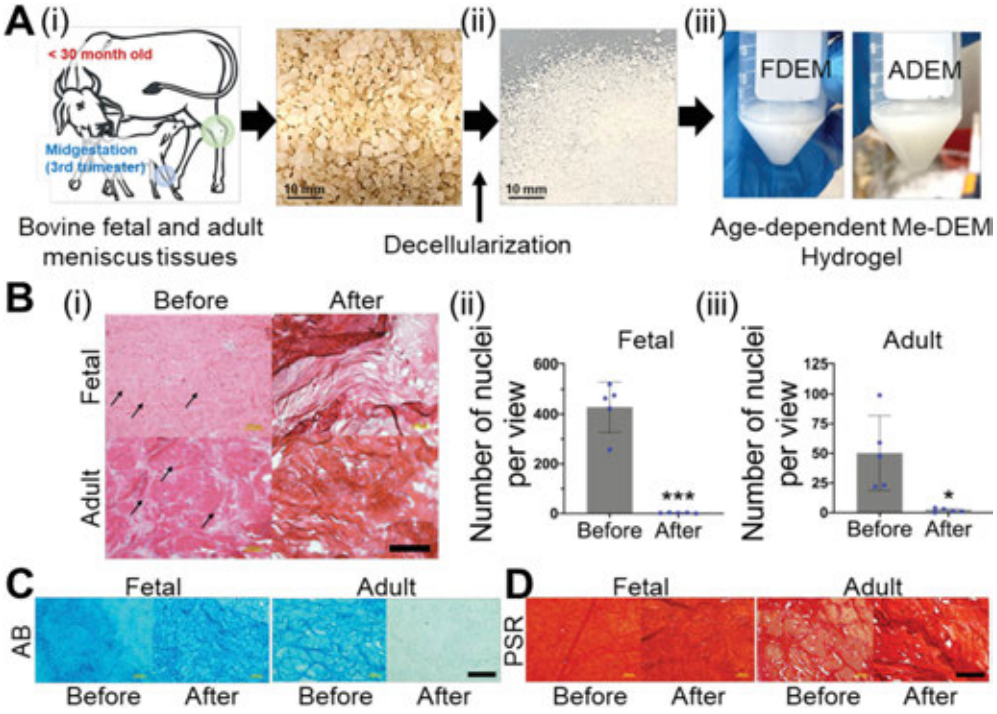

Figure 1. (A) Schematics showing the preparation of age-dependent Me-DEM hydrogels: (i) chopped bovine meniscus tissues, (ii) decellularized meniscus powders, (iii) fabricated FDEM and ADEM hydrogels. **(B)** Representative images of H&E (arrows: nuclei, scale bar: 200 um), quantifications of the number of nuclei in FDEM (ii), or in ADEM (iii) (*$p < 0.05$, ***$p < 0.001$). Representative images of AB **(C)**, and PSR **(D)** (scale bar: 200 um).

meniscus extracellular matrix preservation. The number of nuclei present on the H&E images was counted. Alcian Blue (AB) and Picrocirius red (PSR) staining were performed to confirm the glycosaminoglycan and collagen preservation respectively. The FDEM and ADEM were cross-linked under 37°C for 30 minutes. To prepare the conditioned media, 3.3% DEM [FDEM, ADEM, or FDEM+FEDM (50:50) dissolved in fresh chemically defined (CM) media (v/v)] was incubated under 37°C for 5 days.[8] Juvenile bovine meniscal fibrochondrocytes (MFC, P2) were seeded and cultured on tissue culture plates in basal growth media for 24 hours and the media were replaced with CM media (Ctrl), chondrogenic media CM containing TGF-β3, or the donor age-dependent (FDEM or ADEM) conditioned media (Figure 2A). Cells stained by Phalloidin were imaged at day 3, and the cell areas were quantified by ImageJ. Super-resolution STORM imaging (using ONI) and analysis of histone-H2B (H2B) organization were carried out to determine nano-scale chromatin organization at day 1.[8] Gene expression level of Collagen type-I (Col-I), Aggrecan (ACAN), and ADMST in the MFCs at day 5 were determined using RT-PCR. A short-term MTT assay was performed at day 5 to assess cell proliferation. In addition, at day 5, the PSR staining and 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) staining were performed to determine protein expression from the cells.

Results

The loss of nuclei in the FDEM or ADEM tissues after the decellularization was confirmed by H&E staining (Figure 1B). While no change in GAG contents in the FDEM after the decellularization was observed, the process decreased the GAG contents in the ADEM (Figure 1C). PSR confirmed that collagen was preserved in both groups (Figure 1D) after the decellularization. When the MFCs were cultured in the conditioned media (FDEM or ADEM), increases in cell areas were observed in all DEM-based conditioned media conditions (Figure 2B). Interestingly, changes in the nano-scale chromatin condensation status and gene expression in MFCs were dependent on the media conditions. Compared to the Ctrl media condition, the treatment of TGF-β3 (10.9%), or FDEM (2.3%) increased the chromatin condensation status, while the treatment of ADEM significantly decreased chromatin condensation (by 7.7%) in MFCs (Figure 2C). In addition, the treatments of DEM enhanced the Col-1 expression in MFCs with a significant increase in the FDEM condition, while the media condition decreased the ACAN expression (Figure 2D). Interestingly, the changes in gene expression with the TGF-β3 treatment significantly opposite to those with the DEM treatments (Figure 2E). The expression level of ADMST (which is a marker of ECM remodeling) in the FDEM group was higher than that in the ADEM or the FADEM group (Figure 2E). Finally, the cell proliferation levels were significantly lower in all DEM

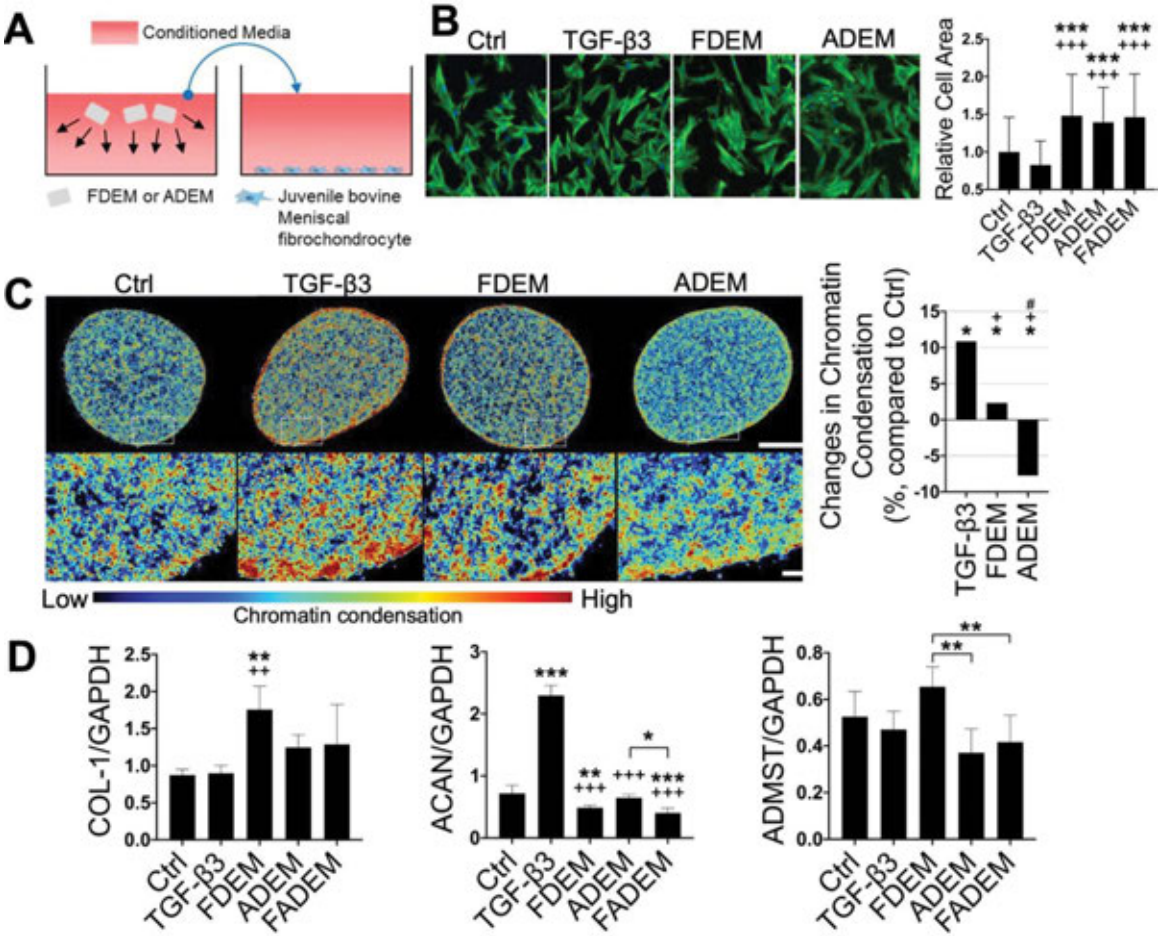

Figure 2. (A) Schematic of in-vitro cell culture in conditioned media. **(B)** Representative F-actin images and relative cell area of MFC cultured for 3 days in conditioned media (FEDM: fetal ECM, ADEM: adult ECM, FADEM: fetal + adult ECM (50:50) (n = 50), **(C)** H2B STORM analysis and quantification of chromatin condensation in MFCs [Scale bar: 1 μm (top) and 500 nm (bottom)], **(D)** Gene expression of COL-1, ACAN, and ADMST at 5 days (n = 5, *: $p < 0.05$ vs. Ctrl, **: $p < 0.01$ vs. Ctrl, ***: $p < 0.001$ vs. Ctrl, +: $p < 0.05$ vs. TGF-β3, ++: $p < 0.01$ vs. TGF-β3, +++: $p < 0.001$ vs. TGF-β3, #: $p < 0.05$ vs. FDEM, ##: $p < 0.01$ vs. FDEM, ###: $p < 0.001$ vs. FDEM).

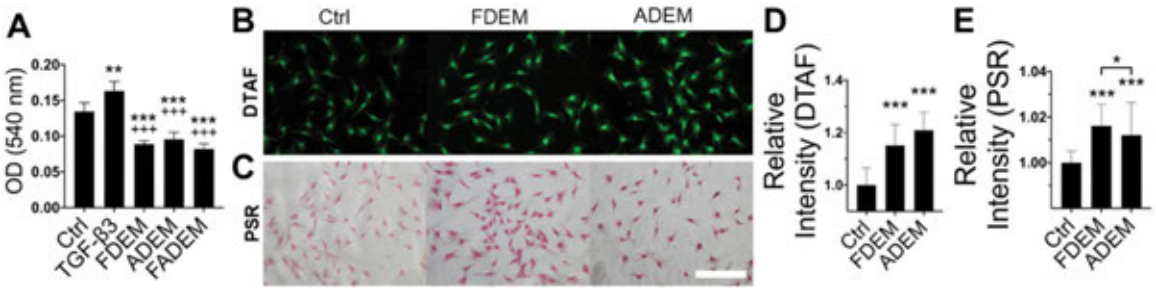

Figure 3. (A) MTT assay result (n=5). Representative images of DTAF **(B)** or PSR staining **(C)**, Scale bar: 200 μm. Quantifications of the relative intensity of the DTAF **(D**, n = 25**)** or PSR staining in MFCs **(E**, n = 25**)** (*: $p < 0.05$ vs. Ctrl, **: $p < 0.01$ vs. Ctrl, ***: $p < 0.001$ vs. Ctrl, +: $p < 0.05$ vs. TGF-β3, ++: $p < 0.01$ vs. TGF-β3, +++: $p < 0.001$ vs. TGF-β3).

groups than in other groups (Figure 3A). However, through DTAF and PSR staining, we found that MFCs in the FDEM and ADEM groups produced more ECM and collagen than MFCs in the Ctrl group (Figs. 3B-E).

Discussion

In this study, we have shown that the treatments of DEM regulate nano-scale chromatin organization, gene expression, proliferation, and matrix production in MFCs. Interestingly, the DEM effects on MFCs are donor age-dependent, and are significantly different from the TGF-β3 treatment. Ongoing studies are focused on elucidating the molecular mechanisms of the donor age-dependent effects of DEM on MFCs using mass spectrometry (to investigate changes in ECM components with the meniscus development) and RNA-seq (to examine how age-dependent ECM impacts transcriptome profiling in MFCs) for their therapeutic potential.

Acknowledgements

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