

Multiplexed Regulation of Meniscal Cell Phenotype and Biomarker Expression Among Matrix-Synthetic Populations

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INTRODUCTION: Meniscus injuries can result in considerable pain and disability, and the 1 million+ meniscus tears annually represent the most common intraarticular knee injury. Current treatments, namely suture repair and meniscectomy, can result in scarring, damage to surrounding tissues, and heightened risk of osteoarthritis. Regenerative engineering offers a promising alternative treatment strategy for meniscus injuries. However, the inner zone of the meniscus is thought to be non-regenerative due to reduced vascularization and a relatively low cell to extracellular matrix (ECM) ratio. Tears in this region often arise as radial tears, oriented perpendicular to the circumferential collagen fiber network. This type of tear completely disrupts the integrity and alignment of the fibers which perform the critical force distribution functions of the tissue. Thus, there is a clear motivation to investigate the identities and abilities of endogenous meniscal cells to deposit and organize new ECM in the early stages of functional inner meniscus regeneration. *While the overall structure and basic cell phenotypes in the meniscus are known, there are no established definitions of distinct meniscus cell populations.* Recently, our group has addressed this gap by performing single-cell RNA sequencing (scRNA-seq) and subsequent analysis of pediatric (ages 12-19) meniscus samples (Fig. 1A). Clustering analysis generated novel meniscus cell subpopulations and revealed differential expression of genes related to ECM production and organization within functionally distinct clusters. The largest of these populations is the fibroblast-like matrix synthetic cells which differentially express genes encoding for Col1, MMP2, and, notably, the cell surface protein **MXRA8** (Fig. 1B). While this gene and its protein product have not been previously characterized in human fibrocartilage, its known relation to integrin binding and cell adhesion suggest that it could be a promising marker for meniscal cells with significant capacity for matrix remodeling and synthesis after injury. Due to the abundance and complexity of ECM within the meniscus, we hypothesize that fibroblast-like matrix synthetic cells with significant MXRA8 expression are primed for regenerating these tissues after injury. Therefore, our objectives for this study were to 1) characterize expression patterns of meniscal cell biomarkers on a range of 2D *in vitro* cell culture substrates, 2) validate the utility of matrix-synthetic biomarkers for isolating functionally distinct populations of human meniscus cells with fluorescence activated cell sorting (FACS), and 3) regulate and quantify the functional capacity of matrix-synthetic meniscal cells to synthesize new ECM by varying cell culture substrates and controlling their adhesion mechanism.

METHODS: Human tissues analyzed by scRNA-seq include inner meniscus samples acquired during partial meniscectomies and discoid saucerization (n=4 female, n=5 male, IRB Exemption #STUDY00000746). NovaSeq analysis was performed at 50,000 reads per cell. Clustering analysis, UMAP generation, and differential gene expression analysis were performed with the Seurat v5 toolkit (RStudio). *In vitro* experiments were performed using a single human cell source (male aged 17, right lateral meniscus, passage 4) cultured and fixed at 2 days, 5 days, and 7 days on tissue culture polystyrene (TCPS) multi-well plates. TCPS coating conditions included non-coated control, pre-coated rat tail collagen I (Millipore), 10 mg/mL human hyaluronic acid (HA, Lifecore Biomedical MW 500 kDa in PBS, overnight incubation), or 10 ug/mL human fibronectin (Corning in water, overnight incubation). FACS experiments were performed at the University of Washington Cell Analysis Facility (UW CAF) with the BD FACSAriaIII, FlowJo software, and UW CAF protocols. FACS experiments included unstained, single stain control, and double stain experimental samples, and results were visualized by Floreada. Endpoint immunostaining experiments were performed using standard 2D protocols (1^o antibodies 1:500 dilution, 2^o antibodies and small molecule stains 1:1000 dilution). Deposition of nascent synthesized proteins was visualized by DBCO labeling via a modified fluorescent noncanonical amino acid tagging (FUNCAT) technique at all timepoints. Immunostained and fluorescently labeled meniscus cells were imaged by confocal fluorescent microscopy (Leica SP8) and processed with FIJI.

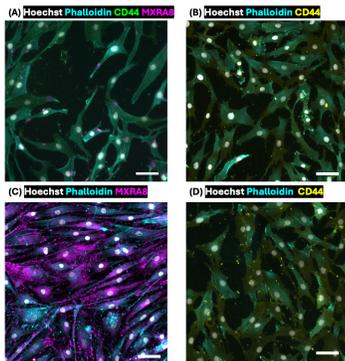


Fig. 2. Immunostaining meniscus cells cultured one week *in vitro* on various 2D substrates. Representative images of heterogeneous human meniscus cells on varying substrates. (A) Non-coated TCPS. (B) Human Fibronectin 10ug/mL. (C) Rat-tail Collagen I (Millipore). (D) Human hyaluronic acid 10mg/mL. scale bar = 50um

RESULTS: Most cells comprising the UMAP were defined as either matrix-synthetic fibroblasts (Cluster 0, COL1A1⁺) or matrix-synthetic chondrocytes (Cluster 1, COL2A1⁺). Analysis of DEGs yielded cell surface biomarkers of interest for matrix-synthetic populations, including MXRA8 and PDGFRB (Cluster 0), TNFRSF11b (Cluster 1), and CD44 (ubiquitous). Cryopreserved human meniscus cells were cultured, fixed, and immunostained with primary antibodies targeting matrix-synthetic biomarkers and stains targeting nuclei (DAPI/Hoechst) and actin filaments (Phalloidin). Unsorted (heterogeneous) cells showed typical adherent fibroblast-like morphology on non-coated TCPS and fibronectin substrate (Fig. 2A-B), while collagen I and HA substrates produced cells with aligned and blebbing morphologies, respectively (Fig. 2C-D). Positive staining of biomarkers was confirmed in unsorted cells, with evenly distributed membrane signal of PDGFRB and CD44 and concentrated and intense patterns of MXRA8 staining (Fig. 2). For the FACS pilot experiment, DAPI was used as a control stain, while MXRA8 expression determined positive selection of cells (Fig. 3A). Sorted cells were cultured alongside unsorted controls on non-coated TCPS, and DBCO fluorescent labeling after 1 week of culture showed that MXRA8⁺ cells produced more nascent ECM than the heterogeneous population (Fig. 3B). Ongoing experiments will assess differences in biomarker expression and nascent matrix production between sorted and unsorted meniscal cells cultured on the various substrates described above. In addition, further work will include administration of CD44 inhibitor Verbasocid and integrin inhibitor Echistatin to focus specifically on each of the major cell-ECM adhesion mechanisms.

DISCUSSION: This work presents novel characterization of human meniscus cells *in silico* and *in vitro*. Because the tissues analyzed by scRNA-seq originated from meniscectomy or saucerization, our analysis lacks true non-injured controls. Despite this limitation, the distinct cell populations identified in these samples, namely the matrix-synthetic fibroblast and chondrocyte cells, support our framework for ECM-centered meniscus regeneration to treat acute injuries. Identification of key biomarkers for these subpopulations within cultured meniscus cells supports the applicability of our analysis for future functionality studies. Furthermore, successful pilot FACS studies suggest that MXRA8 may be a key marker for meniscal cells especially primed for ECM production. Finally, characterizing the cell phenotypes and biomarker expression patterns with both highly targeted cell populations in concert with biologically relevant substrates can heavily inform the influences of environmental cues on innate cell functions.

SIGNIFICANCE/CLINICAL RELEVANCE: These studies support our pursuit of distinct, targeted cell populations which could most effectively regenerate injured meniscus tissues, and the impact of this work is further amplified by focusing on a poorly studied and frequently affected patient population.

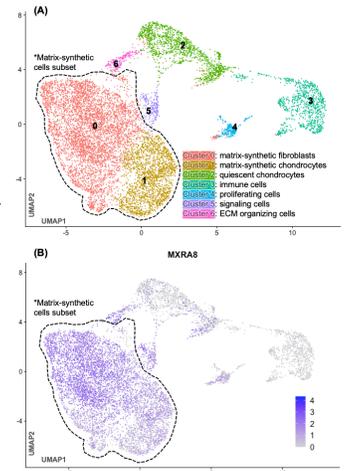


Fig. 1. scRNA-seq analysis results. (A) UMAP plot of human meniscus cell populations (10508 cells total). (B) Heatmap of MXRA8 expression intensity across all clusters.

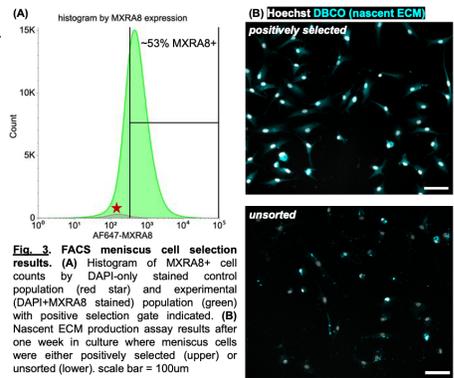


Fig. 3. FACS meniscus cell selection results. (A) Histogram of MXRA8+ cell counts by DAPI-only stained control population (red star) and experimental (DAPI+MXRA8 stained) population (green) with positive selection gate indicated. (B) Nascent ECM production assay results after one week in culture where meniscus cells were either positively selected (upper) or unsorted (lower). scale bar = 100um