Decellularized meniscus (MEND) as a biomaterial that supports stem cell invasion and chondrogenesis

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DISCLOSURES: PMG and RG are inventors on a non-provisional patent application for MEND technology.

INTRODUCTION: Cartilage damage affects 25 million people each year, arising from trauma, sports injuries, and wear and tear **[1]**. Mesenchymal stem cells (MSCs) are a frequently used cell source for tissue engineered cartilage repair because they have improved proliferation rates and are more readily accessible for clinical translation compared to chondrocytes **[2]**. However, MSCs have limitations in their chondrogenic capacity, and even when cultured in 3D hydrogels, matrix secretion outcomes can be non-uniform throughout the construct with non-robust chondrogenesis **[3]**. Given the pro-regenerative capacity of ECM scaffolds, we have previously shown how incorporating cartilage-derived ECM in hydrogel constructs can promote better chondrogenesis **[4]**. Taking this work several steps further, in the Gottardi lab we have developed a new scaffold based solely on decellularized porcine meniscus (MEND), which has sufficient mechanical properties for cartilage repair in the airway and adequate porosity to allow for native cell infiltration both *in vitro* and *in vivo* **[5]**. Notably, MEND is based on the selective removal of elastin from meniscal cartilage which preserves the bulk of its mechanical properties, creating a wealth of channels for re-cellularization. In this study we aim to (i) show that MEND can undergo recellularization with MSCs, and (ii) investigate whether MEND possesses an intrinsic pro-chondrogenic potential for MSCs. To achieve this goal, we seeded MEND with varying densities of MSCs to compare the chondrogenic response of MSCs in MEND against that of MSCs in 3D hydrogels with controlled composition such as methacrylated type I collagen (ColMA) and methacrylated gelatin-hyaluronic acid (GelMA/HAMA).

METHODS: ***MEND Fabrication:*** Porcine derived menisci were cut into 0.5-1mm radial sections, subjected to 4 freeze/thaw cycles (1h) followed by pepsin/acetic acid incubation (24h, 37C) and elastase digestion (24h, 37C) for selective enzymatic removal of elastin. Then, a 6 mm biopsy punch was used to extract cylinders from the red zone region of each section. MEND cylinders were soaked in cell culture medium containing 20% FBS for 24h at 37C before seeding. ***MEND Cell Seeding:*** Human bone marrow derived MSCs at P5 were used (n=3 donors). MEND cylinders were placed in a 24 trans-well, and MSCs suspended in 1% FBS medium were added directly on top of MEND at densities of either 300K, 400K, 600K, or 800K. Medium containing 20% FBS was added to the bottom well to establish a serum gradient across MEND. Media were renewed every 2 days. At day 5, a cell viability assay with Calcein AM was conducted to assess MSC distribution on the top and bottom surface. ***Hydrogel constructs and pellet controls:*** MSCs were suspended at 10 million cells/mL in either ColMA (8 mg/mL, 1M NaOH, lithium phenyl-2,4,6-trimethylbenzoylphosphinate, 10X PBS) or GelMA/HAMA (5% methacrylated gelatin, 2.5% methacrylated hyaluronic acid, 0.15% lithium phenyl-2,4,6-trimethylbenzoylphosphinate). For positive control of chondrogenesis, MSCs were centrifuged to form pellets (200K cells/pellet). ***Analysis of Chondrogenesis:*** To compare the level of chondrogenesis in each condition, all constructs were cultured for 21 days in chondrogenic differentiation medium (Fluorobrite DMEM, 10µg/mL Insulin-Transferrin-Selenium, 40µg/mL L-proline, 2% PSF, 50µg/mL Ascorbic Acid (AA), 10ng/mL TGF-β3) with medium renewed three times per week. Then constructs were analyzed by histology (Safranin-O and Alcian Blue for GAGs, Picrosirius Red with polarized light microscopy (PLM) for collagen, Van Gieson's for elastin), immunofluorescence (IF, for collagen I and collagen II), RT-qPCR (*ACAN, SOX9, COL1A1, COL2A1*), and biochemistry (total DNA/collagen/GAG content, and ratio of collagen/DNA and GAG/DNA).

RESULTS SECTION: ***Chondrogenic Potential of MSC seeded hydrogels:*** ColMA and GelMA/HAMA constructs exhibited non-uniform and variable matrix secretion over 21-day chondrogenesis, and pellet controls followed similar trends (**Figure 1**). Safranin-O and Alcian Blue stains resulted in dis-homogenous matrix in pellets and ColMA constructs relative to day 0 timepoints (data not shown), with co-localization of GAGs, collagen I, and collagen II in the remainder of the constructs. Sporadic expression in ColMA gels could be influenced by cell-mediated contraction that occurred throughout the chondrogenic culture period. GelMA/HAMA scaffolds appear more uniform across histological analyses, but the presence of hyaluronic acid might be a confounding factor for Safranin O stains. Furthermore, no difference in collagen II staining was detected between days 0 and 21 and methodological improvements are in progress to better assess matrix production within these constructs. ***MEND fabrication:*** Structure of MEND and formation of channels was assessed by H&E and Van Gieson's staining, and the comparison to native meniscus showed full removal of elastin and decellularization (**Figure 2, left**). MEND constructs resulted in an average porosity and channel diameter of 8% and 8 µm, respectively. This allowed for successful MSC seeding of MEND over 5 days and was confirmed by a cell viability assay and by DAPI staining for cell distribution (**Figure 2, right**).

DISCUSSION: Our initial data suggest that MEND is uniformly pervaded by channels, ideal for MSC seeding, which was successfully achieved. When seeded in pellets (positive controls) and in the two hydrogels, MSCs underwent chondrogenesis but with dis-homogeneous matrix secretion, as expected based on previous literature. Analysis of differentiation across all constructs by RT-qPCR and biochemistry is currently in progress. Notably, MEND contains both collagen I and collagen II as well as some hyaluronic acid. A limitation to this study is that ColMA, chosen as a collagen I only scaffold to assess the influence of MEND's main ECM component, undergoes rapid cell-mediated contracture upon seeding (within 3-4 days), creating an additional challenge to distinguish secreted vs. scaffold collagen I, which we plan to overcome using non-canonical amino acids (ncAA) to directly tag newly synthetized collagen.

SIGNIFICANCE/CLINICAL RELEVANCE: MEND demonstrates promise in providing a scaffold with composition close to that of cartilage and with an abundance of channels that allows it to be easily repopulated by cells, matching key requirements for clinically relevant articular cartilage engineering. Furthermore, assessing the extent to which MEND supports a robust chondrogenic phenotype of MSCs may open the way to complementing microfracture.

REFERENCES: **[1]** CODE Tech., 2017. **[2]** Le *et al.*, *J. of Tissue Eng.*, 2020. **[3]** Cote, A.J., *et al.*, *Nature Comm.*, 2016. **[4]** Rothrauff, B.B. *et al.*, *J. of Tissue Eng. & Regen. Med.*, 2018. **[5]** Gehret *et al.*, *BioRxiv*, 2022.

ACKNOWLEDGEMENTS: Support from the Children's Hospital of Philadelphia Research Institute, Fontaine Fellowship, NIH P30 AR069619, and T32-AR007132. Thanks to Ryan M. Friedman for ColMA protocols and Stephanie Fung for useful advice.

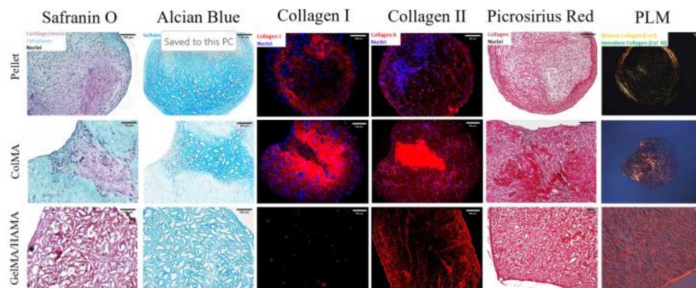

Figure 1 *Histological and IF images of constructs and pellets at 21 days of chondrogenesis.*

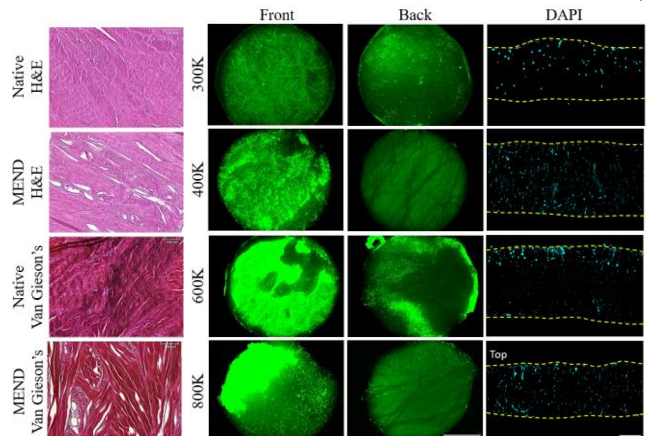

Figure 2 *H&E and Van Gieson's images of native meniscus and MEND (**left**) and Calcein AM/DAPI images of various MSC seeding densities into MEND (**right**).*