Deciphering Avascular Tissue Regeneration in the Human Knee Meniscus via In Vitro Models

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INTRODUCTION: Regeneration of avascular, fibrous tissues like the knee meniscus is rarely achieved. Despite this, a meniscus variant, known as a discoid, can regenerate in the avascular region following resection. Importantly, patients ranging from 5 to 50 years old experienced regrowth, indicating a unique regenerative mechanism despite any impact of aging. The discoid meniscus has two main structural tissue hallmarks: disorganization of collagen fibers and increased proteoglycans between these fibers. The most significant barrier in researching this regenerative motif is a lack of mechanistic models, with no commonly used animal models exhibiting a discoid. *Thus, an in vitro model is critical to understand the mechanisms by which the discoid is regenerating.* **The objective of this study is to build a model that can re-capitulate the *in vivo* hallmarks of the human discoid to interrogate the regenerative motif observed in case studies and begin to investigate potential mechanisms derived from scRNA-sequencing in that model**. The hypothesis is that discoid cells cultured on 3D scaffolds will produce extracellular matrix (ECM) with significantly less alignment and higher amounts of proteoglycans compared to non-discoid meniscal cells and that key transcription factors identified from network analysis will alter this deposition motif. To accomplish this, we utilized our labs expertise in electrospinning, with ECM scale mimetic fibers (micron sized) of either aligned, or unaligned nature. These fibers were then utilized to create three dual-layer construct groups: aligned homotypic, heterogenous, or unaligned homotypic. These fiber orientations mimic the native collagen alignment of discoid (unaligned with random orientation) and non-discoid (aligned and anisotropic). The heterogenous group serves to mimic not only an in-between phenotype (with both discoid and non-discoid mimetic ECM) but also the external, superficial layer of the meniscus where there is a random, unaligned course of fibers on the outside that gives way to aligned fibers underneath. In addition to the mimetic fibers, we performed single cell RNA sequencing (scRNA-seq) and ran the resulting data through high density weighted gene co-expression network analysis (hdWGCNA) to identify genes that may be driving the discoid phenotype.

METHODS: Aligned and unaligned electrospun fibers were used to induce meniscus-mimetic extracellular matrix deposition. Polycaprolactone 5 ± 1 µm fibers were electrospun in chloroform utilizing either static (unaligned) or rotating collection (aligned) (15 kV, 30 cm distance, 800 RPM rotational velocity). Fibers were fused to create dual-layered constructs and secured to induce mechanical boundaries (**Figure 1A**). Primary human cells from saucerization and partial meniscectomy surgeries (IRB approved) were utilized for culture (n = 6 (2 male; 4 female); ages 11-17) split between discoid and non-discoid. Alcian blue staining and 1,9-dimethylmethylene blue assay were utilized for total proteoglycan content. Immunostaining and picrosirius red staining with polarized light were utilized for collagen deposition assessment. Single cell RNA sequencing (scRNA-seq) and hdWGCNA were utilized to generate targets for differences in ECM expression between human discoid and non-discoid meniscal cells. Single cell RNA-sequencing was performed and analyzed in Seurat, nGene > 250, nUMI > 500 and mitochondrial percent < 0.6. Briefly, genes from matrix synthetic discoid cells were utilized to create 7 clusters of similar expression, one of these clusters significantly overlapped with genes specific to discoid from scRNA-seq. hdWGCNA parameters soft power = 7 and metacells = 25. Transcription factors with the highest connectivity coefficients were pulled for literature analysis.

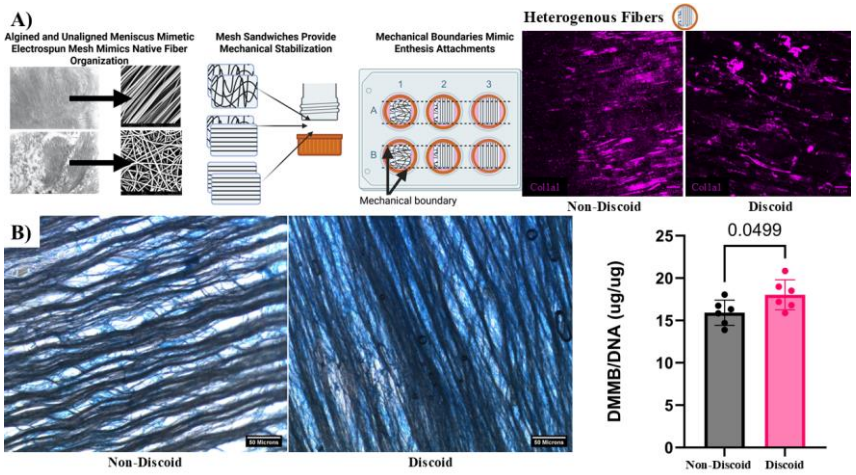

Figure 1. Overall diagram of 3D culture setup, including representative SEM images of aligned and unaligned fibers. Col1a1 immunostaining in 3D scaffolds in heterogenous fibers comparison between discoid and non-discoid (A). Representative alcian blue staining of non-discoid and discoid cells on heterogenous 3D scaffolds. Dimethylmethylene blue assay quantification of sulfated glycosaminoglycan content in 3D scaffolds (B).

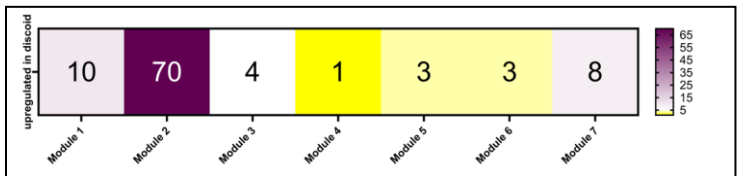

Figure 2. Heatmap of gene co-expression clusters from matrix synthetic discoid cells with the number of genes that are >1.5 log2fold change upregulated in the discoid meniscus samples

RESULTS: On heterogenous dual-layered fibers, non-discoid cells produced aligned collagen whereas discoid cells produced disorganized collagen (**Figure 1A**). Also, discoid cells on aligned, single layer fibers with mechanical boundaries showed a significant accumulation of proteoglycans as well as a higher content of sGAGs from the DMMB assay compared to non-discoid cells (**Figure 1B**). From scRNAseq and hdWGCNA analysis, a co-expression module that overlapped significantly with discoid specific genes (**Figure 2, Module2**) was identified with NR4A1 as a top transcription factor. Interestingly, NR4A1 is known to be involved in collagen regeneration. Current work is focused on a lentiviral vector containing an shRNA for NR4A1 to determine this gene's impact on discoid matrix deposition and growth *in vitro* after transduction.

DISCUSSION: Excitingly, our *in vitro* system with discoid cells re-capitulates the accumulation of proteoglycans and altered deposited collagen alignment observed in the discoid meniscus and will be used to determine the mechanism driving discoid cell contribution to disorganized ECM. Further, the discoid-specific transcription factors identified with hdWGCNA can be utilized further to determine specific genes that might be involved in discoid specific regulation of the extracellular matrix. Future directions include direct interrogation utilizing viral vectors to downregulate NR4A1.

SIGNIFICANCE/CLINICAL RELEVANCE: This work represents the first model (*in vitro* or otherwise) of the human discoid lateral meniscus, a necessary step to probe a mechanism driving regrowth after saucerization. Further, the creation of an *in vitro* model for aligned collagen deposition utilizing human cells allows interrogation of meniscus matrix deposition, beyond the application to the discoid meniscus, and will be used to develop regenerative strategies for the meniscus.