Consistent self-organized emergence of hyaline cartilage in hiPSC-derived multi-tissue organoids

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Disclosures: T.D.O, B.L., A.V., M.T. (Officers in Sarcio Inc). F.T. (Consultant for Sarcio Inc.), Y.W.C. (Employee of Sarcio Inc.). H.I.H. (N), M.L. (N), J.E.A. (N), N.C.M. (N), B.M.O. (N), P.A.L. (N)

INTRODUCTION: Existing methods for producing hyaline cartilage from human induced pluripotent stem cells (hiPSCs) are hindered by several limitations, including complexity of culture conditions, phenotypic instability, and batch-to-batch variability. The objective of this study was to develop a robust, consistent, and xenobiotic-free method to generate hyaline cartilage through harnessing self-organization of multi-tissue organoids (MTOs).
METHODS: A multi-step, xeno-free culture method was implemented to differentiate hiPSCs into MTOs. MTOs were derived from hiPSC line 1024, which were first expanded on vitronectin in E8 medium, then induced to aggregate using a microgel in ultra-low attachment plates before being transferred to a bioreactor for maturation. MTOs were then cultured in defined conditions for up to 15 weeks, with hyaline cartilage emergence noted by 8 weeks. Cartilage differentiation was assessed via immunohistological analysis for cartilage-specific matrix components. The molecular profile and gene expression changes within MTOs over time were characterized using independent bulk RNA sequencing (bulk-seq) at weeks 8, 11, and 15. Single-cell RNA sequencing (scRNA-seq) was used to investigate cellular heterogeneity and differentiation trajectories within the MTOs. Statistical significance for gene expression analysis was set at $p<0.01$.
RESULTS: Histological analysis at 12 weeks confirmed robust cartilage formation, demonstrated by pronounced immunohistochemical staining for collagen type II and aggrecan (Fig. 1). Bulk-seq analysis findings were consistent with maturation of the cartilage phenotype over time, with upregulation of key cartilage-specific genes such as *COL2A1* and *ACAN* ($p<0.01$) by week 15 while hypertrophic markers (*COL10A1* and *MMP13*) were not noticeably different. The gene expression profile of week-15 MTOs closely resembled that of developing human cartilage (Fig. 2). Single-cell RNA sequencing identified that chondrocytes were the majority cell type (78.5 ± 9.1%) with minimal off-target differentiation and pluripotent cell population (Fig. 3). Further analysis showed limited expression of pluripotency markers, with 6.94 ± 1.65% $Oct4^+$ cells and 17.54 ± 4.68% $SSEA4^+$ cells across batches.
DISCUSSION: This study presents a robust, xeno-free method for generating hyaline cartilage using hiPSC-derived MTOs. The use of both bulk-seq and scRNA-seq provided a thorough validation of the chondrogenic differentiation process. Findings demonstrate a stable cartilage phenotype, limited off-target differentiation, and a developmental progression that mimics native tissue development. These results are consistent with a highly promising approach for future cartilage tissue engineering and translational studies.
SIGNIFICANCE/CLINICAL RELEVANCE: Our novel, xeno-free method produces high-quality hyaline cartilage from hiPSCs through MTO intermediaries overcoming key challenges in the field, and by doing so, presents a promising platform for cartilage tissue engineering and potential clinical applications.
ACKNOWLEDGMENTS: We acknowledge Paula Overn and Katalin Kovacs, PhD (University of Minnesota Masonic Cancer Center Comparative Pathology Shared Resource) for histology and analysis support. We also thank the University of Minnesota Genomics Center for generating sequencing data and the Minnesota Supercomputing Institute for providing computing resources.

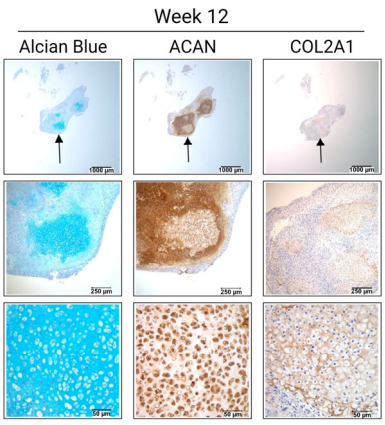

Figure 1: MTOs at 12 weeks show maturing cartilage with Alcian blue positive matrix separating chondrocytes, moderate diffuse staining for type II collagen (COL2A1), and diffuse aggrecan (ACAN) labeling. Arrows indicate areas magnified in the second and third rows. Size bars = 1000 µm, 200 µm, 50 µm for top, middle and bottom rows, respectively.

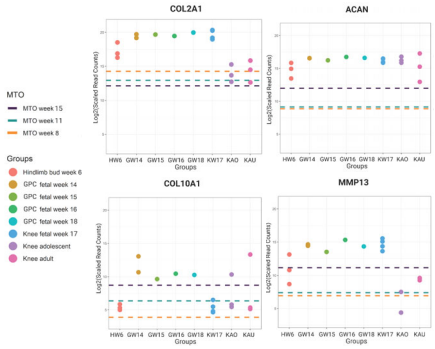

Figure 2: Representative comparisons of marker transcripts (*COL2A1, COL10A1, MMP13, ACAN,*) in MTOs and human lower limb bud cartilage. MTOs – multi-tissue organoids; HW — hindlimb bud week; GW– growth plate chondrocytes fetal week; KW — knee fetal week; KAO — Knee adolescent; KAU — knee adult.

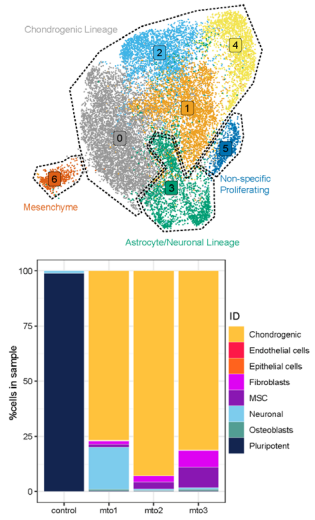

Figure 3: Uniform Manifold Approximation Projection (UMAP) and quantification of SingleR algorithmic labeling of the unique cell populations found across MTOs at the single cell level in comparison to undifferentiated hiPSC control based on the Human Primary Cell Atlas (HPCA) reference.