Continuous Expansion of Human Cruciate Ligament Cells for Ligament Tissue Engineering

Tarek Klaylat[1,2], Paul Martineau[1,2], Showan Nazhat[3], Derek Rosenzweig[2], Rahul Gawri[1,2]

[1]Regenerative Orthopedics and Innovation Laboratory, McGill University, Canada, [2] Division of Orthopedic Surgery, Department of Surgery, McGill University, Canada, [3]Unit of Materials Engineering, Department of Mining and Materials Engineering, McGill University, Canada
tarek.klaylat@mail.mcgill.ca

INTRODUCTION: Anterior cruciate ligament (ACL) ruptures are amongst the most common musculoskeletal injuries [1]. Due to biomechanics and poor blood supply, ligament tissues fail to heal and regenerate after injury properly. Thus, the current standard of care is ACL reconstruction surgery (ACL-R) using tendon grafts of autogenic or allogenic origins. Nevertheless, ACL-R is associated with high failure, complications, and revision rates. Therefore, there is an unmet clinical need for alternative solutions. Tissue engineering regenerative medicine (TERM) applications for ligament tissues is an emerging field, using scaffolds, bioactive compounds, and cell-based therapies. Bioengineered ligament grafts constitute one type of such application. Cell-seeded bioengineered constructs are superior to ones without cell seeding [2]. Nevertheless, uncovering the optimal source of cells for ligament tissue engineering applications remains a challenge. Stem cells from diversified sources have been examined with limited success. Using primary ligament cells for TERM applications for ligament regeneration has dual limitations of limited cell numbers and loss of phenotype (dedifferentiation) on cell passaging. In the current study, we investigate cell number expansion using a biaxial strain device (cellarator) that provides a continuously expanding growing surface for primary ligament cells, thereby expanding primary cell populations without the need for passaging and the resulting dedifferentiation.

METHODS: Human ligament tissues were obtained from patients undergoing ACL-R surgeries per the institutional ethics board-approved protocols and receiving informed consent. Primary human ligament cells were isolated from resected surgical samples by collagenase tissue digestion. Silicone inserts for the cellarator device were chemically activated and coated with collagen type 1 as per established protocols [3]. To match the cell growth surface, Petri dishes of sizes 35mm, 60mm, and 100mm were coated with silicone elastomer and treated the same way as the silicone inserts [3]. Isolated human ligament cells were counted and seeded on the cellarator device with a 10-day continual stretching protocol. Coated 35mm Petri dishes were seeded at the same seeding density, and cells were trypsinized and passaged onto 60mm and 100mm dishes when the silicone dish mounted on the cellarator device reached 60mm and 100mm surface area, respectively. At the end of the stretching protocol, the cells on the cellarator device were still in passage P1, whereas the cells on the surface area matched (100mm) coated Petri dish were in P3. Cells were imaged at each passaging time point (cellarator and static dishes) to assess cell alignment (dispersion index) and morphology (circularity index) using the ImageJ software. qPCR was performed to determine the relative gene expression of ligament markers collagen type-I and III, tenascin C, and scleraxis. Gene expression data were reported using the ΔΔCt method. Metabolic activity was assessed using AlamarBlue assay. For normally distributed datasets, student t-tests, and appropriate ANOVAs with multiple comparisons (Tukey) were performed. For non-parametric datasets, appropriate non-parametric tests such as Mann-Whitney U Test or the Kruskal-Wallis Test were used. Statistical analyses were conducted using GraphPad Prism 10 software.

RESULTS: Cell morphology data shows that continuously expanded primary ACL cells showed a more spindle-like morphology and parallel arrangement (lower dispersion index) than conventionally cultured and passaged ACL fibroblasts (**Figure 1**). Similarly, continuously expanded primary ACL cells maintained their growth rate, while conventionally cultured and passaged ACL fibroblasts showed an altered growth rate (**Figure 2A**). In addition, continuously expanded primary ACL cells displayed a higher metabolic activity compared to their passaged counterparts (**Figure 2B**). Our gene expression analysis results show a declining trend in the transcriptional expression of ACL fibroblast markers collagen type-I, III, and VI, scleraxis, decorin and tenascin C from P0 to P3. However, continuously expanded ACL fibroblasts (at P1) had a significantly higher gene expression (3 to 5-fold changes) of ligament-specific markers collagen type III and VI, scleraxis, and tenascin C compared to their conventionally cultured counterparts (at P3) (**Figure 3**).

DISCUSSION: Our results demonstrate that continuous expansion is a unique technique to expand primary human ACL fibroblast populations for tissue engineering applications for regenerating ligament tissue, while impeding unwanted dedifferentiation and maintaining the phenotype of these cells. Our results align with previous findings relative to the application of this culture technique to the expansion of hMSCs [4] and primary bovine chondrocytes [3]. Limitations of this study are the low sample size and the lack of diversified methods used to characterize the phenotype of the cells in culture. We plan to increase the sample size to enhance the statistical power of our results and conduct sex-based analyses by acquiring more patient samples and employing other techniques such as western blots, immunostaining, and functional ELISA to validate our gene expression results. Furthermore, we aim to seed these cells on bioscaffolds and assess their ability to produce more robust ligament tissue for in vivo implantation in a rabbit model of ACL injury and repair.

SIGNIFICANCE/CLINICAL RELEVANCE: Continuous expansion would allow us to expand populations of primary ACL cells from patients to seed them on bioengineered ligament scaffolds to be used in ACL-R surgery while avoiding dedifferentiation. This would enhance surgical outcomes and the quality of life of individuals and populations at risk ACL rupture such as athletes.

REFERENCES: [1] Sanders *et al* 2016 PMID: 26920430, [2] Bi *et al* 2021 PMID: 33981343, [3] Rosenzweig *et al* 2012 PMID: 22738340, [4] Majd et *al* 2009 PMID: 18974211.
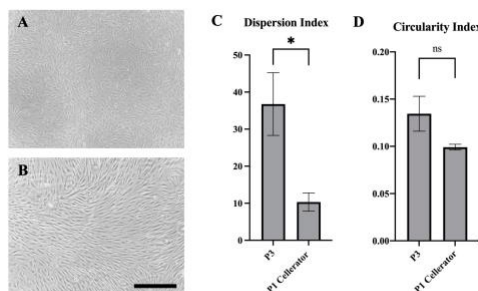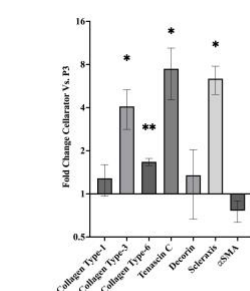
Figure 1: *Morphological assessment of human ACL cells expanded using conventional culture and continuous expansion techniques.* (A) P3 (B) P1 Cellarator (C) Dispersion index (D) Circularity index. Scale Bar represents 50 μm . Mean ± SEM,* p-value<0.05, n=4
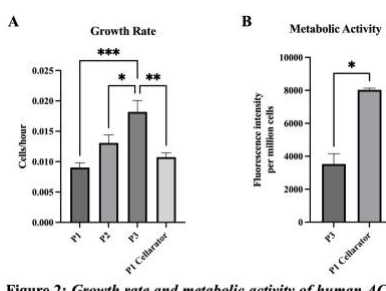
Figure 2: *Growth rate and metabolic activity of human ACL cells expanded using conventional culture and continuous expansion techniques.* (A) Growth rate, n=7 (B) Metabolic activity normalized to cell number, n=3. Mean ± SEM,* p-value<0.05,** p-value<0.01, *** p-value<0.001

Figure 3: *Gene expression of human ACL cells expanded using conventional culture and continuous expansion techniques.* Fold changes in gene expression of P1 Cellarator were normalized to P3 which was fixed to 1. Mean ± SEM,* p-value<0.05, ** p-value<0.01 n=7