Ligamentogenic Differentiation of Human Bone Marrow-Derived Stem Cells Under Near Physiological Culture Conditions for Tissue Engineering Applications

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INTRODUCTION: Ligament injuries are amongst the most common musculoskeletal injuries [1]. Due to biomechanics and poor blood supply, ligament tissues fail to properly heal and regenerate after injury. Thus, the current standard of care is reconstruction surgery. Nevertheless, ligament reconstruction surgeries suffer from high failure rates, complications, and revision rates [2], thus creating an unmet clinical need for alternative solutions. Fabricating implantable bioengineered ligament grafts is one potential application but still faces the challenge of non-integration at the graft site. Cell-seeded bioengineered constructs are superior to their cell-free counterparts. Nevertheless, the optimal source of cells and culture conditions for bioengineering ligament grafts remain a challenge. Stem cells from various sources, bone marrow (*hBM-MSCs*) and adipose-derived stem cells, have been used for ligament graft bioengineering with limited success. Ligament tissue and the articular joint space have lower oxygen tensions than arterial and venous levels due to their inherent hypovascularization, which also results in a reduced nutrient supply, namely glucose. These conditions are crucial for optimal tissue physiology and the integration of bioengineered grafts. In this study, we hypothesized that culturing hBM-MSCs under optimized near physiological oxygen tensions and glucose concentrations would enhance their differentiation into the ligamentogenic lineage for use in tissue engineering and regenerative medicine (TERM) applications for ligament tissues.

METHODS: hBM-MSCs (RoosterBio, n=3, male and female) were cultured for 10 days at 37 °C in DMEM supplemented with 10% fetal bovine serum, 1% antibiotic/antimycotic, 5 ng/mL TGF-β, 1 ng/mL bFGF, and 50 µg/mL L-ascorbic acid. Cultures were maintained under three oxygen conditions: atmospheric (20% O_2), intravenous (*IV*, 5% O_2), and intraarticular (*IA*, 2% O_2), each combined with four glucose concentrations: 0.5 g/L, 1.0 g/L, 2.25 g/L, or 4.5 g/L. For 20% O_2, cell-seeded plates were placed in a standard incubator at 37 °C with 5% CO_2. For IV and IA conditions, hypoxia chambers (StemCell Technologies) were sealed and flushed with gas premixes containing 5% or 2% O_2, respectively, and incubated at 37 °C. Media was replaced every 3 days, with hypoxia chambers resealed and reflushed accordingly. After 24 hours of incubation, hypoxia-inducible factor 1α (HIF-1α) expression was examined using immunofluorescence to confirm that the hBM-MSCs were experiencing hypoxia. At the end of the incubation period of 10 days, trypan blue exclusion assay was used to assess cell numbers and viability. RT-qPCR and Western blot were performed to determine the gene and protein expression of ligamentogenic markers. Picrosirius red staining was used to assess collagen matrix deposition. All datasets were tested for normality, and appropriate statistical tests were performed accordingly.

RESULTS SECTION: Our results confirmed an increase in the expression of HIF-1α in hBM-MSCs cultured under 5% and 2% O_2 tensions compared to those cultured at 20% O_2 tension. Our data show that 5% O_2 tension does not affect the viability of hBM-MSCs, whereas 2% O_2 tension slightly decreases it. We observed increased cell proliferation under near physiological culture conditions. Gene and protein expression analysis reveals that 2% O_2 tension combined with 1g/L glucose seems to promote the highest expression of ligamentogenic markers collagen type 1 and 3, tenascin C, and scleraxis compared to other tested combinations **(Figure 1)**. Collagen matrix deposition evaluated using picrosirius red staining confirmed these findings **(Figure 2)**.

DISCUSSION: These findings suggest that near physiological oxygen tensions and glucose concentrations enhance the ligamentization potential of hBM-MSCs cultured in culture media with TGF-β, bFGF, and L-ascorbic acid. These results align with previous findings on the effects of hypoxic culture conditions on ACL-derived cells [3]. Furthermore, we aim to combine these cells with dense aligned collagen gels to develop bioengineered ligament constructs to be used for ligament reconstruction.

SIGNIFICANCE/CLINICAL RELEVANCE: Optimizing culture conditions to promote the differentiation of hBM-MSCs toward a ligamentogenic lineage would enable their application in the development of bioengineered ligament grafts for reconstructive purposes. This potentially provides a sustainable and reliable solution to the challenge of optimal cell sourcing for cell-based ligament tissue engineering applications, thus bridging the gap towards implantable bioengineered ligament substitutes.

REFERENCES: [1] Sanders *et al* 2016 PMID: 26920430, [2] Nagaraj *et al* 2019 PMID: 30905996, [3] Kowalski *et al* PMID: 26236651

ACKNOWLEDGEMENTS: This work is supported by grants from the Network for Oral and Bone Health Research (RSBO), McGill Regenerative Medicine Network, Quebec Cell, Tissue, Gene Therapy Network (ThéCell), and the Natural Sciences and Engineering Research Council of Canada (NSERC) awarded to RG. TK was supported by doctoral scholarships from RSBO and the Fonds de Recherche du Quebec – Nature & Technology (FRQ-NT).

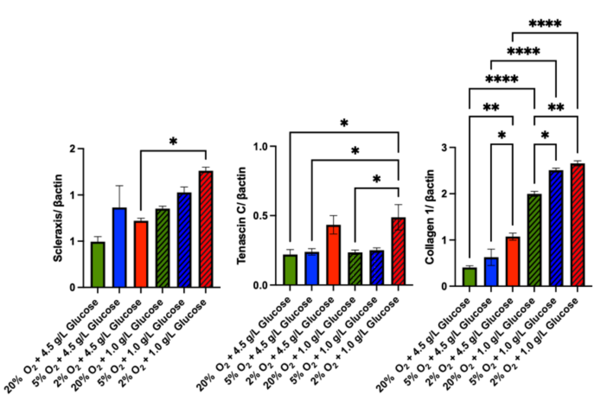

Figure 1: ***hBM-MSCs protein expression of ligamentogenic markers under near physiological culture conditions.*** n=3, Mean ± SEM, *p-value < 0.05, **p-value < 0.01, ****p-value < 0.0001.

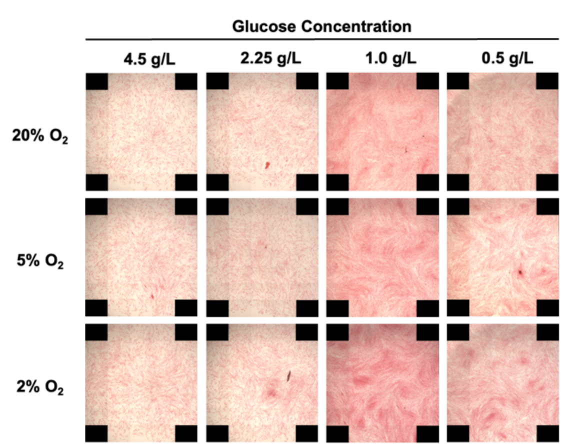

Figure 2: Picrosirius red staining of *hBM-MSCs under near physiological culture conditions.*