

Differentiating Human Bone Marrow and Adipose-Derived Stem Cells Towards Ligamentogenic Lineage Using Physiological Oxygen Tensions 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 have high failure, complications, and revision rates [2]. Therefore, there is 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 ones without cell seeding. 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 (hAD-MSCs), 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, and it is critical for optimal tissue physiology and bioengineered graft integration as the grafts grown in standard non-physiological tissue conditions face “adverse conditions” upon implantation. In this study, we hypothesized that culturing hBM-MSCs and hAD-MSCs under optimized near physiological oxygen tensions would enhance their differentiation into the ligamentogenic lineage for use in TERM applications for ligament tissues.

METHODS: hBM-MSCs and hAD-MSCs (RoosterBio) were cultured for 10 days at 37°C on 100 mm tissue culture Petri dishes in DMEM supplemented with 10% Fetal Bovine Serum, 1% Antibiotic/Antimycotic, 5ng/mL TGF-β, and 1ng/mL bFGF under three different oxygen tensions: atmospheric and standard cell culture oxygen tension (N, 20% O₂), mild hypoxia (MH, 5% O₂), severe hypoxia (SH, 2% O₂). Cell-seeded culture plates were placed in a standard incubator with 5% CO₂ at 37°C for atmospheric oxygen tension. For mild and severe hypoxia, hypoxia incubator chambers (StemCell Technologies) were sealed, and flushed with 5% O₂ and 2% O₂ gas premixes respectively and placed inside an incubator at 37°C for the duration of the experiment. The media was changed every 3 days, and the hypoxia chambers were resealed and refilled. At the end of the incubation period of 10 days, trypan blue exclusion assay was used to assess cell numbers and viability, AlamarBlue assay was performed to evaluate the differences in metabolic activity, and qPCR was performed to evaluate gene expression of ligament/tendon markers. Gene expression data were reported using the ΔΔCt method. All datasets were tested for normality and appropriate statistical tests were performed accordingly. Statistical analyses were conducted on GraphPad Prism 10.

RESULTS SECTION: Our data show that 5% oxygen tension does not affect the viability of hBM-MSCs and hAD-MSCs, whereas 2% oxygen tension slightly decreases the viability of hBM-MSCs. Growth rate analyses reveal that lower oxygen tensions do not affect cell growth and population doubling. AlamarBlue assay readings indicate a significant increase in the metabolic activity of hBM-MSCs under 2% and 5% oxygen tensions and hAD-MSCs under 5% oxygen tension. Finally, qPCR results indicate higher levels of collagen type-1, tenascin C, and scleraxis expression in hBM-MSCs under 2% oxygen tension. Gene expression analysis for the same markers in hAD-MSCs was inconclusive, and no statistically significant difference was observed.

DISCUSSION: These findings suggest that hypoxic culture conditions enhance the ligamentization potential of hBM-MSCs cultured in culture media with TGF-β and bFGF. These results align with previous findings on the effects of hypoxic culture conditions on ACL-derived cells [3]. Our findings on hAD-MSCs could be explained by their low ligamentization potential discussed in the literature [4]. Nevertheless, further analyses such as Western Blots, immunofluorescence, and mitochondrial activity assays will be conducted to elucidate the bioenergetic mechanisms elicited by hypoxic culture conditions in hBM-MSCs. Lowering serum and glucose concentrations will also be investigated with physiological oxygen tensions to expand the current study and optimize the culture conditions. Thereon, we aim to culture and mature our bioengineered cell-seeded ligament grafts under these optimized near-physiological conditions to ensure robust tissue generation and priming to the intraarticular conditions before implantation to ensure graft integration.

SIGNIFICANCE/CLINICAL RELEVANCE: Elucidating adequate and optimal culture conditions for the differentiation of hBM-MSCs and hAD-MSCs into the ligamentogenic lineage would allow us to use these cells in bioengineered ligament grafts for ligament reconstruction. Moreover, priming and maturing these cell-seeded bioengineered grafts under near-physiological conditions could potentially enhance their integration at the graft site and adaptation to the intraarticular conditions, thus lowering revision rates and enhancing the patients’ quality of life.

REFERENCES: [1] Sanders *et al* 2016 PMID: 26920430, [2] Nagaraj *et al* 2019 PMID: 30905996, [3] Kowalski *et al* PMID: 26236651, [4] Eagan *et al* 2012 PMID: 21953999

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