Replicating Endochondral Processes Through the Induction and Control of Periosteal Cell Differentiation

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Introduction: Endochondral Ossification (EO) occurs during embryonic development of mammals, and it is involved in bone lengthening and healing, postnatally. It has previously been shown that Periosteum-derived Cells (PDCs) play an important role during fracture healing through EO, thus making this cell source a potential candidate for clinical applications. In this study we employed a micromass culture system to study chondrogenic and hypertrophic differentiation of human PDCs (hPDCs) using different cocktails of Growth Factors (GFs). A RhoA/ROCK signalling inhibitor (Y27632) was used to modulate cytoskeletal tension and enhance differentiation. The main goal is to achieve intrinsic control over the rates of chondrogenic and hypertrophic differentiation of hPDCs *in vitro*.

Material and Methods: hPDCs were expanded in DMEM supplemented with 10% FBS and 1% Sodium Pyruvate before being harvested and cultured in micromass at high density. After adhesion, micromasses were cultured for 7 days in basal chondrogenic medium (Low Glucose DMEM + 100 nM dexamethasone, 100 µM ascorbate-2-phosphate, 40 µg/mL Proline, ITS and 20µM Y27632) supplemented with 8 different combinations of GFs according to a multifactorial design. From day 7 until day 14, micromasses were cultured in hypertrophic differentiation medium (α-MEM + 10% FBS, 50µg/mL Ascorbic acid, 10mM β-glycerophosphate, 30 ng/ml Thyroxine and 10 µM Y27632). Subsequently, they were analysed at several time points (3,7,11 and 14 days) for structural changes (histology), glycosaminoglycan (GAG) deposition (alcian blue), matrix mineralization (alizarin red), metabolic activity (presto blue), and marker gene expression (qPCR).

Results: hPDCs cultured in a micromass system supplemented with Y27632 displayed higher deposition of matrix GAGs, and showed enhanced capacity to resist cytoskeletal tensions promoted by TGFβ1. This was indicated by their ability to remain attached to the culture surface under serum free conditions. Following stimulation with different combinations and concentrations of GFs (BMP2, BMP6, GDF5, FGF2 and TGFβ1), distinct degrees of chondrogenic and hypertrophic differentiation were achieved. Gene expression analysis showed enhanced expression of chondrogenic genes Sox9 and Col2 until day 7, followed by the onset of hypertrophy, which was indicated by the expression of ALP. Moreover, persistence of Runx2 and Col10 expression until the last time point (day 14) was found. Histology indicated the presence of distinct matrix compositions depending on the different growth factors combinations used in culture.

Discussion: Collectively our data suggests that, under the given conditions, BMP2 is the strongest inducer of the initial stages of chondrogenic differentiation. However, supplementation with BMP2 and BMP6 during the first 7 days is essential for the induction of a mineralized matrix at later time points. Thus, it is apparent that the mechanism for the differentiation of the initial stage (chondrogenesis) is crucial for further development of a hypertrophic mineralized matrix. Also, low concentrations of GDF5, FGF2 and TGFβ1 play important roles for the final outcome. The analysis of marker gene expression further indicates that micromasses were stimulated to follow the natural endochondral ossification cascades.

Conclusion: Herein, we demonstrate the chondrogenic and hypertrophic potential of hPDCs through the use of a specific culturing protocol including micromass culture and specific media components. GF stimulation effectively controls the two different stages of differentiation, providing an excellent basis for the development of a semi-autonomous construct for endochondral bone formation.