Altered Epigenetic Status and Cellular Mechanosensitivity in Human Chondrocytes During *in vitro* Expansion

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Introduction: Articular cartilage poses a significant challenge due to its limited intrinsic healing capacity. Autologous chondrocyte implantation (ACI) holds great promise as a surgical intervention for the treatment of cartilage defects **[1]**. However, a significant challenge in ACI lies in the dedifferentiation of chondrocytes during *in vitro* expansion, leading to the loss of their phenotype. This dedifferentiation involves the downregulation of canonical markers defining the cartilage phenotype, limiting the therapeutic efficacy of ACI **[2]**. Thus, understanding the mechanisms driving chondrocyte phenotype loss during *in vitro* expansion is pivotal. Moreover, harnessing this knowledge is essential to preserving the original phenotype during the expansion, thereby enhancing the therapeutic efficacy of ACI in addressing cartilage damage. To this end, this study aims to explore the impact of *in vitro* monolayer expansion on the nano-scale chromatin organization, epigenetic status, and cellular mechanosensitivity of human chondrocytes, providing valuable insights for optimizing cell-based cartilage regeneration therapies.

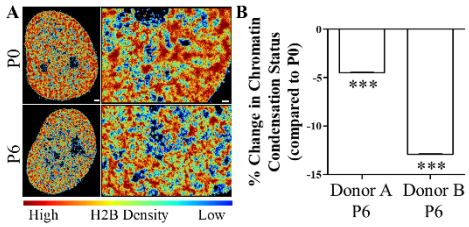

Figure 1. A: Heat maps showing H2B localization density [bars = 1 μm (left) and 200 nm (right), **B:** Quantitative analysis of chromatin condensation (n>15/group, ***: p<0.001 vs. P0).

Methods: Human articular chondrocytes from three donors (Donor A: 46-year-old male; Donor B: 55-year-old male; Donor C: 52-year-old male, provided by LifeLink Foundation) were isolated and expanded on tissue culture plastic up to passage six (P0 to P6) in basal growth media (BM). Super-resolution STORM imaging (ONI) of histone-H2B (H2B) was carried out to assess nano-scale chromatin organization and analyzed using the Nanoimager software (ONI) **[3],** and the chromatin condensation parameter was determined by normalizing Voronoi density of H2B to the mean Voronoi polygon density in a nucleus **[3, 4]**. To investigate alterations in histone modification status during *in vitro* expansion, immunoblotting of acetylation of H3K9 (H3K9Ac, epigenetic activation), tri-methylation of H3K4 (H3K4me3, epigenetic activation), tri-methylation of H3K27 (H3K27me3, epigenetic suppression), and tri-methylation of H3K9 (H3K9me3, epigenetic suppression), and histone-H3 (H3) were performed. For a further comprehensive insight into epigenetic changes associated with the dedifferentiation process, we conducted a histone H3 modification multiplex assay (Epigentek) to screen and measure changes in 21 histone H3 modifications in P0 and P6 cells. Given that chondrocytes are mechano-responsive, we further examine whether chondrocyte dedifferentiation alters their mechanosensitivity. To achieve this, soft (2–8 kPa) and stiff (18–24 kPa) polyacrylamide hydrogels were fabricated. P0 and P6 cells were seeded and cultured for 3 days (**Fig. 3A**). Immunofluorescence data of YAP nuclear to cytoplasmic (N/C) ratio and cell morphology (Phalloidin) were obtained for each stiffness at P0 and P6 using a Widefield microscope (Leica). Statistical analyses were performed using student's t-test or ANOVA with Tukey's post hoc testing.

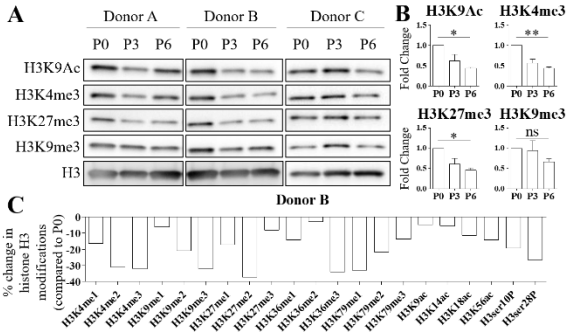

Figure 2. A: Immunoblot of histone markers and **(B)** quantifications at P0, P3 and P6. (normalized to H3, n=3, *: p<0.05, **: p<0.01), **C:** Quantification of changes in 21 histone H3 modifications in P6 cells compared to P0 cells (normalized to H3).

Results: STORM analysis revealed a decreased H2B density in chondrocyte nuclei, indicating that chromatin becomes less condensed with *in vitro* expansion (**Fig. 1B**). This alteration in chromatin structure is intricately associated with changes in histone modification status, influencing gene expression patterns pivotal for maintaining cell identity. Indeed, *in vitro* expansion significantly decreases expression of both activation and suppression of histone-H3 modification markers, indicating a broader yet intricate epigenetic reprogramming during chondrocyte dedifferentiation, potentially correlated with the loss of the specialized chondrogenic phenotype (**Fig. 2B, C**). Chondrocyte culture on soft substrates is recognized for better supporting the maintenance of the chondrogenic phenotype **[5]**. When chondrocytes were cultured on the hydrogels, cells seeded on soft hydrogels exhibited a smaller cell area, lower aspect ratio, and higher circularity compared to those on stiff substrates (**Fig. 3B, C**). Furthermore, dedifferentiated P6 chondrocytes displayed altered mechanosensitivity compared to primary P0 chondrocytes. Notably, when P6 cells were seeded on the hydrogels, they exhibited a larger cell area and more elongated cell morphology compared to primary P0 chondrocytes (**Fig. 3B, C**). More interestingly, the assessment of alterations in YAP nuclear localization levels between chondrocytes cultured on soft and stiff substrates revealed that P6 cells exhibited significantly greater nuclear YAP localization levels on both substrates compared to P0 cells (**Fig. 3C**). This observation suggests that *in vitro* expansion of chondrocytes alters their mechanosensitivity.

Discussion: Our findings demonstrate that the chondrocyte dedifferentiation process associated with *in vitro* expansion changes in chromatin organization, accompanied by alterations in histone modification status in chondrocytes. The decreased expression of both activation and suppression histone modification markers signifies comprehensive and intricate epigenetic reprogramming during dedifferentiation. This process is crucial as it potentially contributes to the loss of the specialized chondrogenic phenotype during their expansion. The increased cell area and elongation, and increased YAP nuclear localization levels in P6 cells on both soft and stiff substrates imply that these cells respond differently to mechanical cues compared to their lower passage counterparts. Taken together, this study illuminates the complex interplay between epigenetic changes, chromatin organization, and cellular mechanosensitivity during the *in vitro* expansion of chondrocytes. Understanding these mechanisms is crucial for improving the efficacy of ACI by preserving the chondrogenic phenotype and enhancing therapeutic outcomes in the treatment of cartilage defects. Ongoing studies are focused on identifying specific roles of different histone modification markers in the regulation of chondrogenic gene expression with ChIP-seq and scRNA-seq, as well as on uncovering the mechanisms that underlie the observed alterations in mechanosensitivity in chondrocytes to enhance the therapeutic potential for translation.

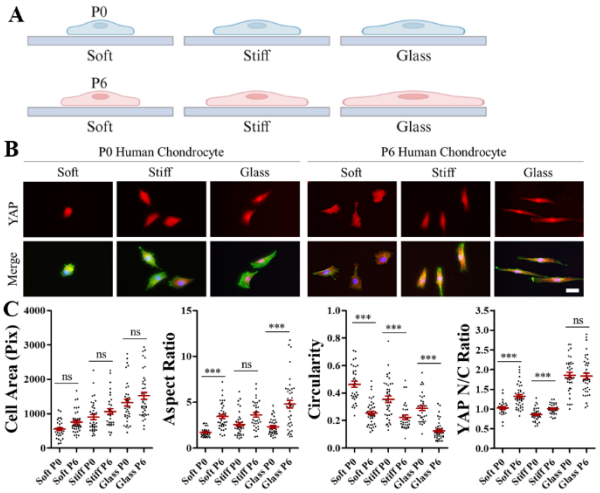

Figure 3. A: Schematic showing experimental setup, **B:** YAP (red), F-actin (green), and DAPI (blue) immunostaining. Scale bar = 30 μm. ***: p<0.001, **C:** Quantifications of cell area, aspect ratio, circularity, and YAP nuclear: cytoplasmic ratios (YAP N/C) (n>30/group).

Significance: By understanding the alterations in epigenetic patterns and mechanosensitivity during chondrocyte dedifferentiation *in vitro*, this study contributes to the generation of chondrocytes with enhanced therapeutic efficacy, offering promising avenues for the treatment of damaged cartilage.

References: **[1]** Roberts+ 2003, *Arthritis Res Ther*; **[2]** Cote+ 2016, *Nat Commun*; **[3]** Heo+ 2020, *Sci Adv*; **[4]** Otterstrom+ 2019, *Nucleic Acids Res*; **[5]** Zhong+ 2013, *J Mol Hist.*

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