STAT1 Inhibition During *In Vitro* Expansion Preserves Chondrocyte Phenotype and Matrix Forming Capacity

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Introduction: Articular cartilage repair remains a major clinical challenge due to the tissue's limited intrinsic healing capacity. Autologous chondrocyte implantation (ACI) is a promising cell-based therapy for cartilage defects **[1]**, but its success is constrained by the rapid dedifferentiation of chondrocytes during their *in vitro* expansion. This process leads to the loss of cartilage-specific markers and diminishes reparative potential **[2]**. Previously, we showed that *in vitro* expansion alters chromatin organization and histone modifications, resulting in changes to chondrogenic gene expression **[3]**. However, the upstream mechanisms that govern these epigenetic and transcriptional shifts remain poorly defined, limiting the ability to maintain a stable chondrogenic phenotype during expansion. To address this challenge, we employed single-cell multiomic analyses to uncover transcriptional and chromatin-level regulators of chondrocyte dedifferentiation. Building on these findings, we further investigated whether targeting these regulatory pathways could enhance the 3D matrix-forming capacity of culture-expanded chondrocytes, thereby improving their utility for cartilage repair.

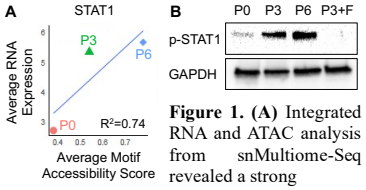

Figure 1. (A) Integrated RNA and ATAC analysis from snMultiome-Seq revealed a strong correlation between STAT1 expression and motif accessibility across chondrocyte passages. **(B)** Western blot analysis showed increased STAT1 protein levels with chondrocyte expansion, which were reduced by Fludarabine (F) treatment at passage 3 (P3).

Methods: *Chondrocyte expansion:* Human articular chondrocytes from four male donors (ages 42, 46, 52, and 55 years; LifeLink Foundation; only male donor tissues were available from the provider) were isolated and expanded on tissue culture plastic from passage 0 (P0) to passage 6 (P6), with or without the treatment of Fludarabine (F), a selective STAT1 inhibitor. During expansion, cells in the treatment group received Fludarabine at 10μM for two consecutive overnight treatments after attachment at each passage. *Transcription factor (TF) identification:* Single-nuclei Multiome sequencing (snMultiome-Seq; RNA+ATAC) was performed on P0, P3, and P6 chondrocytes. Gene expression and chromatin accessibility data were integrated using a weighted nearest neighbor approach, and TFs showing the strongest correlation between gene expression and motif accessibility were identified as candidate regulators of dedifferentiation. *In vitro pellet culture:* Chondrogenic pellets were formed using 2×10^5 P6 cells expanded without (P6) or with Fludarabine treatment (P6+F), with no drug added during pellet culture. Pellets were cultured in chondrogenic medium without (CM-) or with (CM+) TGF-β3. At day 7, RT-PCR was performed to assess chondrogenic gene expression, and histological staining with Alcian blue and Picrosirius red was used to evaluate glycosaminoglycan (GAG) and collagen matrix deposition, respectively (**Fig. 2A**). *FUNCAT assay:* Functional noncanonical amino acid tagging (FUNCAT) was used to assess nascent matrix protein synthesis. P0, P6, and P6+Fludarabine cells were encapsulated in 5 kPa norbornene-modified HA (NorHA) hydrogels at a density of 1×10^6 cells/mL. Azidohomoalanine (AHA) was added as a methionine analog, and at days 7, 14, and 21, samples were imaged to quantify total nascent protein area following click chemistry-mediated reaction with a DBCO-modified fluorophore (**Fig. 3A**). *Statistical analysis:* Data were analyzed using Student's t-test or one-way ANOVA followed by Tukey's post hoc test.

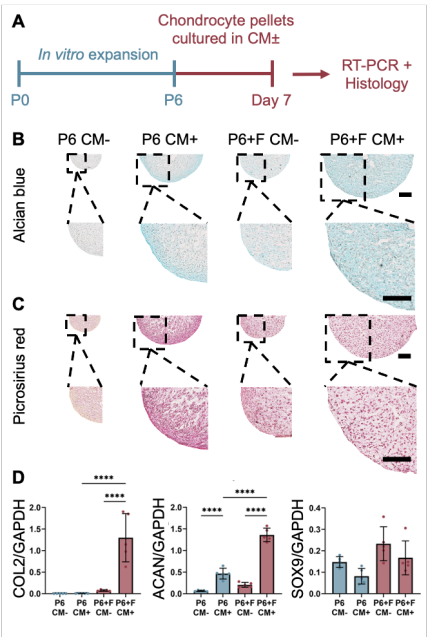

Figure 2. (A): Schematic of pellet culture study design. **(B)** Alcian blue staining of pellet sections showing glycosaminoglycan (GAG) deposition. **(C)** Picrosirius red staining of pellet sections showing collagen deposition. Scale bars: 250μm. **(D)** RT-PCR analysis of chondrogenic gene expression (****$p < 0.0001$).

Results: snMultiome-Seq analysis identified STAT1 as a top transcription factor associated with chondrocyte dedifferentiation, with its gene expression positively correlated with motif accessibility (**Fig. 1A**). Western blot analysis confirmed that phosphorylated STAT1 protein levels increased with dedifferentiation and that Fludarabine, a selective STAT1 inhibitor, effectively suppressed STAT1 phosphorylation (**Fig. 1B**). *In vitro* pellet culture demonstrated that dedifferentiated P6 cells cultured without TGF-β3 (P6 CM-) produced minimal GAG and collagen, while supplementation with TGF-β3 (P6 CM+) increased deposition, though it was restricted it to the pellet periphery (**Fig. 2B, C**). In contrast, P6 cells expanded with Fludarabine (P6+F) exhibited enhanced and more homogeneous GAG and collagen deposition, even without TGF-β3 (P6+F CM-). The strongest and most uniform staining was observed in P6+F pellets cultured with TGF-β3 (P6+F CM+) (**Fig. 2B, C**). Consistent with histological findings, RT-PCR revealed that Fludarabine alone upregulated chondrogenic genes, and that its combination with TGF-β3 markedly increased expression of type 2 collagen (COL2), aggrecan (ACAN), and SRY-box transcription factor 9 (SOX9) (**Fig. 2D**). At the single-cell level, FUNCAT analysis showed minimal nascent protein synthesis in P6 cells compared to unexpanded P0 controls. Fludarabine treatment (P6+F) significantly increased protein production by day 7, and by day 14 (data not shown) and 21, expanded chondrocytes produced levels of nascent proteins similar to P0 cells. Moreover, the spatial organization of nascent proteins in P6+F cells resembled that of P0 chondrocytes (**Fig. 3B, C**).

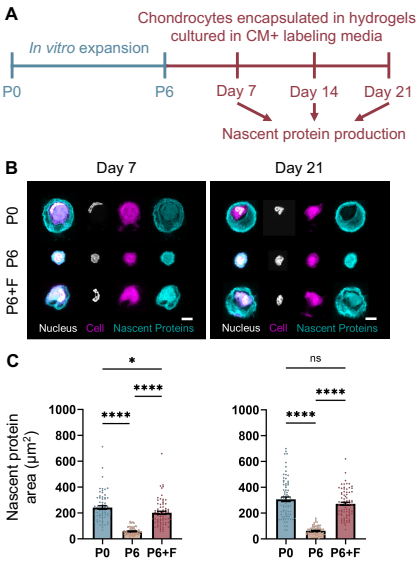

Figure 3. (A) Schematic of FUNCAT study design. **(B)** Representative FUNCAT images showing nascent protein synthesis in chondrocytes encapsulated in hydrogels. Scale bar: 10μm. **(C)** Quantification of nascent protein production at days 7 and 21 (*$p<0.05$; ****$p < 0.0001$).

Discussion: This study provides new mechanistic insights into chondrocyte dedifferentiation, a critical obstacle to the success of ACI for cartilage repair. Through snMultiome-Seq, we identified STAT1 as a central transcription regulator of the transition from differentiated to dedifferentiated states. Pharmacological inhibition of STAT1 with Fludarabine preserved the chondrocyte phenotype, as evidenced by sustained gene expression of chondrogenic markers and enhanced extracellular matrix production in 3D culture. Together, these findings establish STAT1 as a key regulator of chondrocyte dedifferentiation and establish its inhibition as a promising strategy to improve the quality and therapeutic efficacy of chondrocyte-based interventions. Future studies will investigate Fludarabine *in vivo* to further validate its translational potential.

Significance: This study identifies STAT1 as a key regulator of chondrocyte dedifferentiation. Pharmacological inhibition of STAT1 preserved chondrocyte phenotype at both the transcriptional and matrix production levels, suggesting a promising strategy to preserve chondrogenic potential during *in vitro* expansion. Targeting STAT1 may enhance the efficacy of ACI, thereby advancing cartilage repair therapies and improving long-term outcomes.

References: [1] Roberts+, *Arthritis Res Ther 2003*; **[2]** Cote+, *Nat Commun 2016;* **[3]** Zhang+, *ORS 2025*.

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