Chondrocyte Expansion Results in Dedifferentiation Through Alterations in Contractility and Nuclear Engagement

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INTRODUCTION: Modern autologous chondrocyte-based arthritis therapies are effective at treating small cartilage defects [1]. However, the short timeframe for cell proliferation before chondrocyte dedifferentiation becomes detrimental limits the number of available cells. This short 4-week culture period limits treatment of large defects which would require significantly more chondrocytes. In vitro expansion of chondrocytes drives de-differentiation due to cellular engagement with a stiff culture environment that activates cell spreading, nuclear flattening, and cytoskeletal remodeling [2]. One cytoskeletal complex that is impacted by this is the linker of nucleoskeleton and cytoskeleton (LINC) complex. Previous work has identified that components of the LINC complex are lost with de-differentiation of chondrocytes [3] and alterations in expression are observed with a wide range of other differentiation processes [4]. Here, we investigated changes in contractility and the LINC complex during chondrocyte expansion, and tested whether an imbalance in expression contributes to the contractile phenotype with dedifferentiation.

METHODS: Cell Isolation, Culture, and Transfection – Bovine chondrocytes were extracted from bovine articular cartilage of the radiocarpal joint the day of slaughter, by digestion with collagenase (1mg/ml in DMEM) overnight, followed by cell straining and culture on tissue culture plastic (DMEM, 10% FBS, 1% Penicillin Streptomycin) with 1:3 passages once every 7 days. Male cows were used for these initial experiments, due to the higher rates of autologous chondrocyte usage in male patients. For overexpression experiments, a mini-Nesprin2G construct was transfected into expanded chondrocytes using jetPRIME. Immunostaining – Cells seeded overnight on fibronectin coated coverslips (10µg/ml) were fixed with 4% PFA and stained for YAP (secondary Alexa-647 goat anti-mouse) and Alexa-488 phalloidin with DAPI mounting agent nuclear stain. Cell images were acquired on a Zeiss Axio Observer 7 with Colibri7 light source and Hamamatsu Flash 4.0 sCMOS camera using a 20x 0.8NA objective. Cell and nuclear areas were quantified using the analyze particles tool in ImageJ after thresholding images. Nuclear to cytoplasmic ratio of YAP was quantified using a previously described MATLAB script after nuclear segmentation [5]. qPCR – mRNA expression was examined for LINC Complex and chondrogenic genes (Nesprin 2, Lamin A/C, Aggrecan, CTGF, and GAPDH) using iScript and iTaq (Bio-Rad). Traction Force Microscopy (TFM) – 10kPa PDMS gels were spin coated onto Mattek coverslip bottomed dishes, then modified with 100nm diameter fluorescent beads, and coated with fibronectin (10ug/ml) in PBS before seeding cells overnight. A live-cell imaging chamber with 5% CO_2 on a Zeiss Axio Observer 7 was used to capture bead displacements before and after cell lysis (63x 1.4NA), and average traction stress and total force were quantified using a previously described open-source MATLAB script [6]. For overexpression experiments, the RFP tag was used to identify, image, and threshold transfected cells.

RESULTS: YAP was activated in chondrocytes on glass coverslips at every passage but showed slightly higher nuclear localization with passage, as nuclear spread area increased (Fig. 1A-C). Expression of two LINC complex genes decreased across passage number (Fig. 1D-E), with Nesprin 2 having an early drop and Lamin A showing a decrease at late passages. Expression of the proteoglycan aggrecan also decreased with passage (Fig. 1F). The YAP target gene CTGF was expressed across all passages, further confirming the YAP activation results (Fig. 1G). TFM showed a significant increase in average traction stress (Fig. 1H) and total force across multiple passages, correlating with chondrocyte dedifferentiation. A Nesprin-2 overexpression proved effective at reducing the average traction stress of late passage chondrocytes (Fig. 1I), partially reversing the de-differentiated phenotype.

DISCUSSION: Loss of LINC complex components occurred during dedifferentiation of chondrocytes, especially at late passages. Late passage chondrocytes also displayed higher average traction stresses compared to early passage chondrocytes. Nesprin-2 overexpression was used to reverse this loss, in an attempt to maintain cytoskeletal-to-nuclear coupling. This resulted in a reduction in cellular traction forces for these high passage cells.

SIGNIFICANCE/CLINICAL RELEVANCE: Ensuring the appropriate levels of vital LINC complex proteins could lessen the effects of chondrocyte dedifferentiation and has the potential to extend the viable passage-period in which chondrocytes can be expanded for use in arthritis therapies. Limiting the dedifferentiation process such that the cells can remain healthy chondrocytes could increase the expanded cell number for clinical use and allow for widescale restoration of larger defects in arthritic cartilage.

REFERENCES: [1] Jacobi+ *Sports Med. Arthrosc. Rehabil. Ther. Technol.* (2011) [2] Sliogeryte+ *Osteoarthritis Cartilage* (2016) [3] Gosh+ *Biophysical Journal* (2022) [4] King+ *FEBS* (2023) [5] Naha+ *JOR* (2024); [6] Han+ *Nat Methods* (2015);

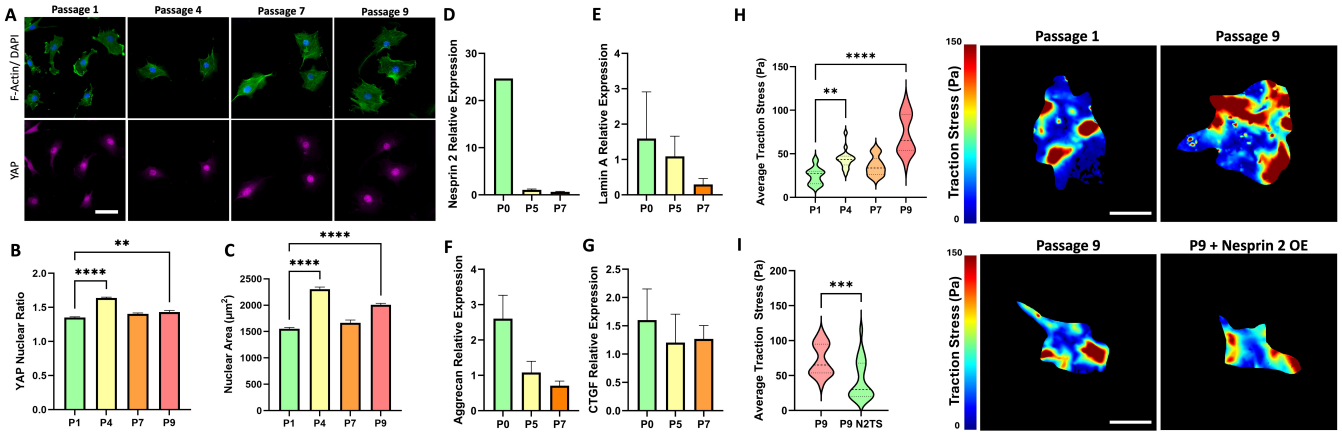

Figure 1: YAP and F-actin/DAPI staining (A), nuclear ratio (B), and nuclear area (C) with passage (Bar graphs indicate means +SEM, n = 50 -100 cells per group. One-way ANOVA with Tukey's post hoc *p<0.1, **** p<0.0001) PCR fold-change relative expression of LINC proteins Nesprin 2 (D) and Lamin A (E), aggrecan (F), and YAP target gene CTGF (G) (Bar graph values are means + SEM, n = 1-3 samples per group from two experiments). TFM cell stress maps and average traction stress for chondrocytes with passage (H). TFM cell stress maps and average traction stress for Nesprin 2 overexpressed chondrocytes at passage 9 (I). (Bar graph values are means + SEM, n = 10 - 20 cells per group. One-way ANOVA with Tukey's post hoc for passage and t-test for transfection *p<0.1, ** p<0.05, **** p<0.0001). Scale bars = 50µm.