# Regulation of Nucleus Pulposus Cell Phenotype Through Actomyosin Contractility and Cell Shape

Gabriella C. Bond[1, 2*], Nadeen O. Chahine[1, 2]
[1]Department of Biomedical Engineering, [2] Department of Orthopedic Surgery, Columbia University, New York, NY (*gb2797@columbia.edu)

**Disclosures:** The authors have no conflicts of interest to disclose.

**INTRODUCTION:** The intervertebral disc (IVD) is a load-bearing tissue, which requires mechanoresponsive cells that can transmit changes in load and microenvironment into internal cues. During intervertebral disc degeneration (IDD), the nucleus pulposus (NP), a typically gelatinous and proteoglycan-rich environment, undergoes matrix catabolism and stiffening. Rho/ROCK, a prominent mechanotransduction pathway, responds to microenvironmental changes by regulating the actomyosin contractility of cells[1]. This also mediates cell shape, where healthy NP cells *in situ* are rounded and have an actin cytoskeleton that is cortical, rather than peripheral and extended when cells are plated in 2D[2]. Prior work has shown that the inhibition of chondrocyte contractility in 2D systems improves phenotype through a decrease in cell area and increase in expression of genes such as aggrecan (ACAN) and collagen II (COL2)[3]. The goal of this study is to explore the changes in NP cell gene expression in the context of actomyosin contractility and cell shape. We hypothesize that NP cell phenotype will be promoted (i) in 2D through inhibition of RhoA by preserving rounded cell morphology and (ii) in 3D through activation of RhoA to increase rounded cell morphology and cortical actin.

**METHODS: Bovine NP cell isolation and culture:** NP tissue was dissected from the lumbar spine of 3 male, juvenile cows. Within each donor, tissue was digested in complete media as previously described[4]. Bovine NP (bNP) cell digest was passed through a 70-µm cell strainer, washed, counted, and grown in complete media. For treatment in 2D, passage 2 cells were plated in a 12-well plate at 200K cells/well. **Alginate Bead Synthesis:** NP cells were suspended at 1 million cells per ml in 1.2% alginate. Beads were created by passing the mixture through a 21G needle dropwise into a bath of 102 mM $CaCl_2$ and allowed to crosslink for 20 minutes. The cells in beads were cultured for 24 hours in complete media before treatment. **Treatments:** Cells in 2D were cultured in the following conditions: (i) untreated, (ii) CT04 (2 µg/ml, RhoA inhibitor, Cytoskeleton Inc) or (iii) CN03 (1 µg/ml, RhoA activator, Cytoskeleton Inc). In 3D, cells were treated with CN03 (1 µg/ml) for 1 day or dosed daily for 7 days. At the end of treatment, cells were lysed for RNA isolation with TRIzol and RNeasy Mini Kit (Qiagen). **Cell staining:** Cells were fixed and stained with Alexa647-phalloidin for actin, anti-pMLC + AF488, and DAPI for nuclei. For imaging flow cytometry, cells were stained with Zombie red for viability. 2D cells were imaged under a confocal microscope and analyzed using cell-profiler to quantify pMLC mean intensity, actin mean intensity, and cellular area. **Bulk RNA Sequencing:** Isolated RNA from cells in 2D were used for bulk RNA sequencing. For each sample, library preparation was completed with STRPOLYA kits and sequenced on Illumina NovaSeq 6000. The variance stabilizing function in DESeq2 was used to normalize gene count data. Differentially expressed genes (DEGs) were identified based on $|\log2FoldChange| \geq 1.0$ and adjusted p value (padj) $< 0.05$. **Gene Expression:** RT-qPCR was conducted to measure expression of extracellular matrix (ECM) markers aggrecan (ACAN) and collagen I (Col1). **Imaging Flow Cytometry:** Cells from 3D culture were analyzed using Amnis ImageStream X Mark II imaging flow cytometer. Cells were gated for in-focus, singlets, live cells, and positive nuclei, and analyzed for pMLC intensity, actin intensity, and cellular area.

**RESULTS:** In monolayer culture for 24 hours, cells treated with CT04 exhibited decreased pMLC and actin intensity, and lower cell area when compared to untreated cells (Figure 1a). Bulk RNA sequencing data highlights distinct clustering of the CT04 and untreated cells, with 1402 DEGs in CT04 vs. untreated NP cells. *GDF5*, *CHST3*, and *MUSTN1* are upregulated with CT04 addition. Out of the 148 enriched reactome pathways, we confirmed that CT04 downregulated Rho related pathways. Additionally, ECM-related pathways were upregulated in CT04 treated cells (Figure 1b). This was further validated by gene expression, where the CT04 treated cells expressed higher levels of *ACAN* and lower levels of *COL1* vs. untreated cells in 2D (Figure 1c). In 3D culture, CN03-treated cells exhibit increased actin and pMLC intensity, while retaining a smaller cell area (Figure 2a). At both 1 day and 7 days of daily CN03 dosing, *ACAN* gene expression levels were increased.

**DISCUSSION:** The goal of this study was to elucidate the NP phenotypic gene expression changes that accompany changes in RhoA signaling in different microenvironments. Decreased cell area signifies a decrease in cell spreading, more closely mimicking NP cell morphology *in situ*. The upregulation of genes involved in chondrogenesis[5-7] including Growth Differentiation Factor 5 (*GDF5)*, Chondroitin 6 Sulfotransferase (*CHST3)*, and Mustang, *MUSTN1*)suggests that CT04-treated cells retain their NP-like phenotype more than their untreated counterparts. This is further supported by the enrichment of ECM proteoglycans, ECM production and Collagen Formation Pathways, along withincreased expression of *ACAN* and decreased expression of *COL1*. CN03 treatment in 2D showed little to no changes, likely because cells in 2D are already highly mechanoactive by attachment to tissue culture substrate (not shown)[3].

The use of 3D alginate culture promotes rounded morphology. Alginate is an advantageous 3D culture substrate for this study because it is bioinert, where changes are directly related to cell shape and decoupled from integrin binding, which can also influence this pathway[8]. Addition of CN03 in 3D further lowers cell area and increases circularity of these cells, likely due to an increase in contractility. While prior studies show that CN03 protects against possible catabolism from NF-kB signaling[4], the current study shows that CN03 to be a modulator of cell phenotype and ECM production.

**SIGNIFICANCE :** The study provides an understanding of how cell-shape and actomyosin contractility can be used to promote NP phenotype. Furthermore, Rho/ROCK could be a potential molecular target for the development of IDD therapeutic and regenerative strategies.

**REFERENCES:** [1] Amano et al, 2010, Cytoskeleton [2] Fearing et al, 2019, FASEB J [3] Hallstrom et al, 2023, MBoC [4] Hernandez, P. A., et al, 2020, Science Advances [5] Coleman, C.M., 2013, Stem Cells Dev [6] Han, J. 2017, OA Cartilage [7] Gersch, R.P., 2010, Bone [8] Neves, M.I, 2020, Front Bioeng Biotech

**ACKNOWLEDGEMENTS:** Supported in part by NIH NIAMS R21 AR080516, R01 AR077760, T32 AR080744.

**Figure 1.**

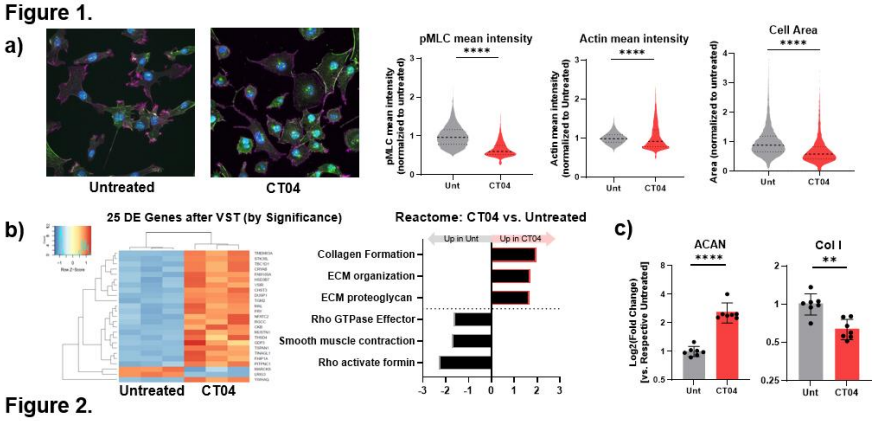


**Figure 2.**

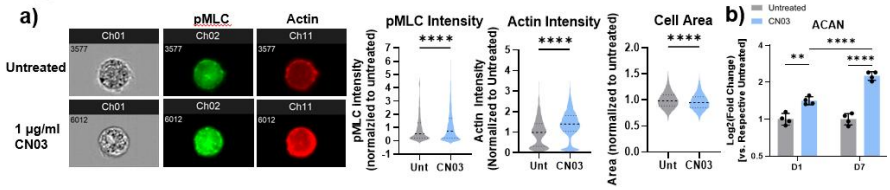


**Figure 1.** CT04-treated bNP cells in 2D have **a)** decreased pMLC, actin, and cell area **b)** enriched ECM-related pathways and **c)** increased ACAN expression and decreased Col1 expression compared to untreated. **Figure 2. a)** 1-day dose of CN03 in 3D leads to increased pMLC and actin intensity but decrease in cell area. b) 1-day and 7-day daily doses of CN03 increase ACAN gene expression. ** $p<0.01$, **** $p<0.0001$