Physiologic TGF-β doses improve cellular strain profiles and pericellular matrix morphology in neocartilage

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INTRODUCTION: Cartilage tissue engineering is a promising osteoarthritis (OA) treatment strategy whereby cell-seeded scaffolds are cultivated to generate neocartilage repair tissues. The long-term survival of neocartilage is dependent on its ability to recapitulate the complex spatial distribution of composition, structure and the material properties of native cartilage, which are requisite for load supporting functionality. Tissue composition includes: 1) an extracellular matrix (ECM), comprised of dense glycosaminoglycans (GAG) enmeshed within a fibrous type-II collagen (Col-II) network, which allows for mechanical load transmission through the tissue, and 2) a sparse arrangement of chondrocytes isolated within an annular Col-VI pericellular matrix (PCM), which modulates load transmission to cells from the ECM. Transforming growth factor beta (TGF-β) has become one of the most prominent mediators for promoting cartilage regeneration due to its strong ability to promote ECM biosynthesis, thus motivating recent efforts to develop innovative scaffold delivery platforms that promote controlled, *in situ* TGF-β delivery to cells during tissue regeneration. Interestingly, the TGF-β delivery profile of these platforms can vary considerably, and we still possess a limited understanding of the optimal dose release rate needed to maximize the quality of regenerating cartilage.

Recently, our group has advanced the novel hypothesis that administration of physiologic doses of TGF-β (0.1-1ng/mL), akin to those presented to cells during native cartilage development[1], can improve cartilage regeneration relative to that achieved with administration of the highly supraphysiologic TGF-β doses (10-10,000ng/mL)[2] that are conventionally used in tissue engineering applications. Our recent work has illustrated the ability of physiologic TGF-β dose administration to improve neocartilage mechanical properties and tissue composition (elevated Col-II relative to Col-I)[3]. Of further note, physiologic TGF-β promotes an isolated chondrocyte morphology, akin to that of healthy native cartilage, in contrast to a clustered morphology induced by supraphysiologic TGF-β, akin to morphology of cells in OA. In this study, we aim to better understand the physiologic implications of cell morphology outcomes on tissue function by examining: 1) cell strain profiles and 2) PCM distribution in neocartilage in response to physiologic (0.3ng/mL) and conventional supraphysiologic (10ng/mL) TGF-β dosing. Here, *in situ* cell strain profiles are evaluated via finite element analysis (FEA) and optical experimental measures.

METHODS: Tissue Constructs: Primary immature bovine articular chondrocytes were seeded in 2% w/v type VII agarose at 30×10^6 cells/mL and cultured in chondrogenic media supplemented with active TGF-β3 at a physiologic (0.3ng/mL) or supraphysiologic dose (10ng/mL) for the initial two weeks or maintained free of TGF-β (0ng/mL). **Mechanical Assessment**: After 56 days, construct E_Y (n=5 per group) was measured in response to 10% unconfined compression. **Cell Morphology**: After 56 days of culture, live constructs (n=4 per group) were diametrically halved and cross-sections were stained with calcein AM and imaged under a confocal microscope. Cellular regions were identified by adaptive Gaussian thresholding and active contouring[4,5], and the fraction of cells in a clustered morphology (cell cluster area fraction [CAF]) versus isolated was determined based on the area and circularity of cell regions[6] using a lab-developed software. **FEA Cell Strains**: An FEA model was implemented in MATLAB to predict stress and strain of cells in native cartilage and TGF-β exposed constructs (0.3 or 10 ng/mL TGF-β) in response to applied physiologic loading (stress=20 kPa). Confocal-derived cell morphology images were binarized and used in the model. **PCM Staining**: Constructs (n=5 per group) were fixed at day 56, paraffin sectioned, and stained for Col-VI (Invitrogen) to detect the PCM. **Experimental Cell Strains**: At day 56, constructs were diametrically halved, calcein AM stained, and confocal imaged before and after the application of a 40% platen-to-platen axial compressive strain using a custom-made apparatus. The engineering strain $(L-L_0/L_0)$ was computed for cellular regions (isolated and clustered, n=3 per group) via ImageJ.

RESULTS: Neocartilage E_Y was enhanced to a similar degree by 0.3ng/mL (629±58kPa) and 10ng/mL (550±75kPa) doses, relative 0ng/mL (179±65kPa). Cell clustering was significantly enhanced by 10ng/mL exposure (CAF=0.79±0.05), relative to 0ng/mL (CAF=0.10±0.01) (Fig. 1A; $p<0.0001$). In contrast, 0.3 ng/mL significantly mitigated cell clustering (CAF=0.18±0.03; $p<0.03$), only marginally above 0ng/mL levels (Fig. 1B). Col-VI staining illustrated a highly organized annular PCM around isolated chondrocytes, akin to native morphology, while 10ng/mL induced a far less organized PCM around clusters (Fig. 1C). Minimum principal strains for ECM and cellular regions were predicted in response to 20 kPa axial compression (Fig. 2A). Models demonstrated that the clustered cellular regions derived from 10 ng/mL exposure exhibited an elevated and more disperse strain distribution (ε=0.33±0.11) relative to more isolated cellular regions derived from 0.3ng/mL (ε=0.21±0.06) or cells in native cartilage (ε=0.21±0.05) (Fig. 2B). Experimental measures of cell strains exhibited similar trends where cell region strains were elevated in large clusters (ε=0.23±0.02), relative to isolated cells (ε=0.07±0.02; Fig 3; $p<0.002$).

DISCUSSION: The dense chondrocyte clusters in response to supraphysiologic TGF-β is a striking outcome when considering their similarity to the morphology of pathologic chondrocyte clusters classically observed in OA. Clusters likely result from TGF-β-induced cell hyperplasia while in the confines of an agarose scaffold that restricts cell expansion. FEA analysis and experimental measures together illustrate an important consequence of this aberrant morphology, whereby clusters exhibit elevated and highly heterogeneous strain profiles in response to physiologic tissue loading. Given the high sensitivity of chondrocytes to strain magnitude, it is reasonable to surmise that clustered cells may lose their ability to respond in unison to physiologic mechanical stimuli. Cell clusters are additionally accompanied by an aberrant PCM morphology that may further exacerbate mechanobiological dysregulation. In contrast, cartilage regeneration in presence of physiologic TGF-β dosing promotes neocartilage with native-matched mechanical properties while maintaining a healthy, more isolated cell morphology. As such, these cells may retain ability to provide a harmonious response to loading, thus better recapitulating the mechanobiological behavior needed to maintain long-term tissue health. In the future, we aim to perform investigations into long-term functional consequences of aberrant cell morphology in neocartilage and examine potential benefits of physiologic TGF-β administration in improving the long-term tissue health.

SIGNIFICANCE: This work advances a paradigm shift illustrating that moderated physiologic TGF-β doses can improve cartilage regeneration, and thus motivating the development of novel scaffold delivery platforms that can achieve sustained physiologic dose delivery to improve clinical cartilage repair.

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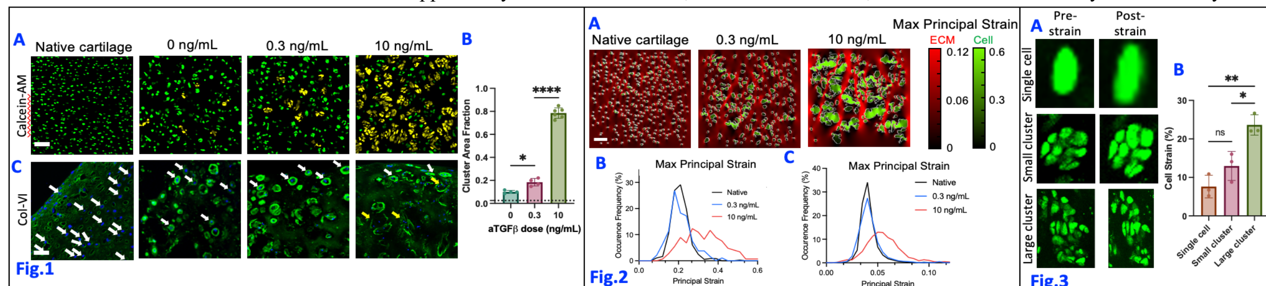

Fig. 1: (A) Representative confocal images showing isolated (green) and clustered (yellow) cell regions. Scale bar: 100 µm. **(B)** CAF of TGF-β supplementation doses. Dashed line: CAF of native cartilage, <0.01. *p<0.03, ****p<0.0001. **(C)** Representative images showing nuclei (blue) and Col-VI (green). White arrow: isolated cells and PCM. Yellow arrow: clustered cells and PCM. **Fig. 2: (A)** FEA model derived color maps of minimum principal strain in cell (green) and ECM (red) regions. **(B, C)** Distributions of minimum principal strain of **(B)** cellular and **(C)** ECM regions. Scale bar: 100 mm. **Fig.3: (A)** Representative images of cellular region of different morphology pre- and post- 40% compressive strain. Single cells: constructs treated with 0.3 ng/mL TGF-β. Small and large clusters are from constructs treated with 10 ng/mL TGF-β; **(B)** Cell strain of different cellular regions: ns p>0.12, * p<0.03, **p<0.002.