Physiologic TGF-β yields functional engineered cartilage while mitigating hypertrophy for expanded chondrocytes

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INTRODUCTION: A challenge in cartilage tissue engineering (CTE) is the generation of neocartilage that recapitulates a native cartilage extracellular matrix (ECM) required for tissue function, while maintaining a stable chondrogenic phenotype. The CTE workflow generally consists of two phases: 1) expansion of cells in 2D monolayer to promote initial chondrogenesis and enhance cell number, followed by 2) *in vitro* cultivation of cell-seeded constructs in 3D, in order to generate neocartilage with a cartilaginous ECM. Transforming growth factor beta (TGF-β) is a prominent growth mediator, supplemented as a chondrogenic priming factor during 2D expansion and stimulator of ECM biosynthesis during 3D construct cultivation. Conventionally, during *in vitro* growth periods, constructs are exposed to highly supraphysiologic doses of active TGF-β (10-100 ng/mL) supplemented in culture medium, representing activity levels that are 10-1,000-fold higher than those present during native development. While these exposure regimens accelerate ECM biosynthesis, they are further associated with promoting features detrimental to hyaline cartilage function, including cell hyperplasia and cell hypertrophy. Recently, we demonstrated that the alternative use of physiologic TGF-β doses (0.1-1ng/mL) for primary chondrocytes yields neocartilage with native-matched mechanical properties while mitigating hyperplasia and hypertrophy, thus supporting the use of physiologic TGF-β for improving neocartilage derived from primary chondrocytes. However, it remains unclear whether conventional TGF-β priming protocols during cell expansion will desensitize chondrocytes to TGF-β, thus reducing their responsiveness to low, physiologic TGF-β doses during in vitro cultivation.

In this study, we aim to examine the effect of physiologic and supraphysiologic TGF-β doses on *in vitro* engineered cartilage development using expanded chondrocytes subjected to varying priming doses of TGF-β during 2D expansion phases. Here we evaluate construct mechanical properties, ECM composition, cell morphology, and hypertrophic gene expression (COL-X and RUNX3). Physiologic TGF-β dosing is administered via: 1) the supplementation of active TGF-β (aTGF-β) in culture medium, and 2) the use of our recently developed bio-inspired latent TGF-β (LTGF-β) scaffolds that enable physiologic *in situ* cell-mediated activation, akin to TGF-β delivery processes to chondrocytes in native cartilage.

METHODS: Chondrocyte priming: Primary immature bovine chondrocytes were subjected to three different cell expansion protocols: Unprimed (control), primed with 0.1ng/mL aTGF-β1 (Primed-0.1), or primed with 1ng/mL aTGF-β1 (Primed-1). Expansion was performed over 3 passages using high glucose DMEM + 1% antibiotic-antimycotic + 10% of fetal bovine serum. The primed groups were additionally treated with 10ng/mL PDGF-ββ and 5ng/mL FGF-2. **Construct *in vitro* cultivation**: After expansion, chondrocytes were encapsulated as cylindrical constructs (∅3×2mm) in 2% w/v methacrylate agarose with or without LTGF-β1 scaffold conjugation as a dose of 150 ng/mL. The unconjugated constructs (n=5 per dose) were exposed to media-supplemented aTGF-β3 for the initial 2 weeks of culture at a physiologic dose (0.3 or 1ng/mL), supraphysiologic dose (10 or 100ng/mL), or maintained TGF-β-free for the entire culture period. **Construct analysis**: At day 42, construct Young's modulus (E_Y) was evaluated in unconfined compression. Construct glycosaminoglycan (GAG) content was evaluated via DMMB assay. Cell morphology was evaluated via live/dead confocal imaging. COL-X and RUNX3 expression was evaluated via qPCR.

RESULTS: For all priming groups, supraphysiologic aTGF-β dose (10ng/mL) induced 12.3-fold to 39.3-fold enhancements in construct E_Y, relative to TGF-β-free ($p<0.0001$), yielding constructs with native-matched E_Y (Fig.1A). Physiologic aTGF-β at 1ng/mL, yielded 8.0-fold to 28.9-fold E_Y enhancements ($p<0.0001$), and aTGF-β at 0.3ng/mL yielded 3.6-fold to 13.6-fold E_Y enhancements ($p<0.05$). LTGF-β scaffolds induced 3.7-fold and 9.8-fold E_Y enhancements for unprimed and Primed-0.1 groups, respectively. However, for Primed-1, E_Y enhancements were reduced. GAG content measures followed similar trends to E_Y (Fig.1B). For all passage groups, supraphysiologic aTGF-β significant enhanced the expression of hypertrophic markers (COL-X and RUNX3) (Fig.2), and induced hyperplasia, as marked by a clustered chondrocyte morphology (Fig 3). In contrast, physiologic aTGF-β mitigated COL-X and RUNX3 gene expression and maintained chondrocytes in a more isolated morphology.

DISCUSSION: While conventional supraphysiologic TGF-β (10 & 100ng/mL) is highly effective at enhancing construct E_Y, it is further accompanied by features detrimental to hyaline cartilage function, including the induction of cell hypertrophy, as marked by elevated COL-X and RUNX3, and cell hyperplasia, as marked by the dense clustering of chondrocytes. Alternatively, physiologic TGF-β (0.3 & 1ng/mL) remains able to significantly enhance construct E_Y, but while mitigating COL-X and RUNX3 expression, and maintaining a more isolated chondrocyte morphology, consistent with the cell morphology in healthy cartilage. Interestingly, physiologic TGF-β doses retain the potential to significantly enhance construct E_Y, even in the aftermath of administration of TGF-β priming during 2D expansion, suggesting physiologic TGF-β can still be used to improve *in vitro* construct development, even for cell populations that require expansion to increase cell numbers. We observe that the benefits of physiologic TGF-β delivery can be achieved via delivery of aTGF-β via media supplementation or via conjugation to the scaffold in its latent form. LTGF−β scaffolds provide cells with a stable reservoir of LTGF-β to undergo activation via cell mediated processes (e.g., integrins), akin to delivery in native cartilage, thus providing cells with physiologic TGF-β activity. Consistent with our prior work on primary chondrocytes, [1-3] LTGF-β scaffolds yield significant enhancement of E_Y for constructs derived from primed chondrocytes.

SIGNIFICANCE/CLINICAL RELEVANCE: The administration of physiologic TGF-β via media supplementation or LTGF-β scaffold conjugation can significantly enhance the mechanical properties of constructs derived from expanded chondrocytes, while mitigating cell hypertrophy and hyperplasia.

REFERENCES: [**1**] Wang+2023 Tissue Eng Part A 31: 56-68. [**2**] Wang+2025 (in press) bioRxiv [**3**] Albro+2015 Biomaterials 77:173-185.

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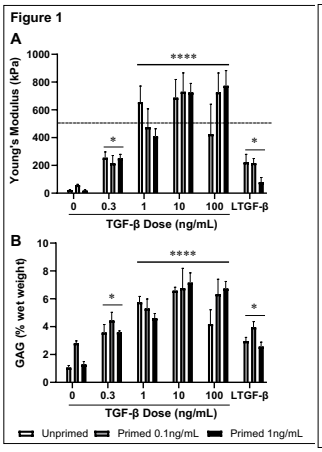

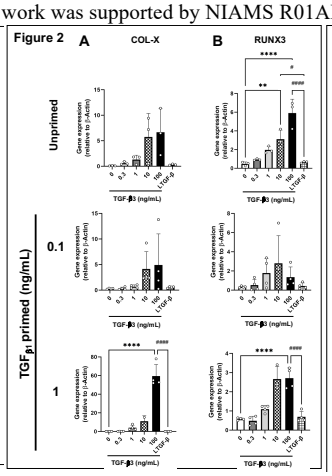

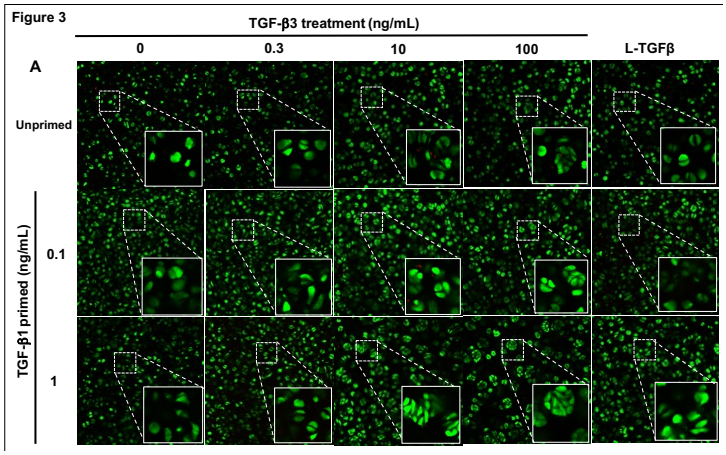

Figure 1. Mechanical property (**A**) and glycosaminoglycans (GAG) content (**B**) at day 42 (n=4-5 per group). *$P < 0.05$ and ****$P < 0.0001$ vs. TGF-β3 untreated. **Figure 2.** COL-X (**A**) and RUNX3 (**B**) gene expressions in the groups at day 42 (n=4 per group). **P < 0.01, ****P < 0.0001 vs. TGF-β_3 untreated, #$P < 0.05$ and ####$P < 0.0001$ vs. TGF-β3 treated with 10 and 100 ng/mL doses. **Figure 3.** Chondrocytes' clustering in active $TGF_{\beta 3}$ treated and L-TGF-β conjugated constructs (n=2 per group).