Synergistic GDF5–CTGF signaling drives efficient, scalable, and off-target-free Syndetome to iTenocyte induction

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DISCLOSURES: The authors have no conflict of interest to declare.

INTRODUCTION: Current cell-based therapies for tendon repair lack lineage specificity, often drifting toward fibroblastic or chondrogenic fates instead of stable tenogenic phenotype. These shortcomings promote fibrosis and lead to poor functional healing. To address this, we developed a scalable, xeno-free differentiation protocol to generate highly efficient and tenogenically committed cells. In this study, we report that context-dependent BMP signaling through GDF5, supported by CTGF as a regulatory mediator of extracellular matrix maturation (ECM)–cell interactions, rather than TGFβ, synergistically induces tenogenesis while expanding the Syndetome (SYN) progenitor-like pool. We further hypothesize that pharmacological activation of mechanosensitivity pathways could mature enable tenogenic ECM in iTenocytes, providing an alternative to complex and time-consuming mechanical stimulation.

METHODS: TGF, BMP, FGF, and Hedgehog signaling pathways were sequentially modulated to achieve Sclerotome (SCL) differentiation from iPSCs in a stepwise manner **(Fig. 1A)**. SCL-specific gene expression was confirmed by qPCR, and dynamic cluster transitions were assessed using single-cell RNA sequencing (scRNAseq) analyzed with the Seurat v5 pipeline. Feature plots were constructed to analyze the shift from iPSC to SCL. Subsequent induction to SYN was performed by reseeding SCL cells and applying TGFβ3, GDF5, and CTGF for 6 days. The iTenocytes were obtained treating SYN cells with GDF5/CTGF combination for 6 days. Immunofluorescence (IF) was used to confirm tenogenic markers presence. Presto Blue viability assay was used to determine the fold change in cell numbers throughout the study. Finally, pharmacological PIEZO1 activation was used to optimize iTenocyte maturation by comparing dynamic mechanical stimulation on scaffolds in mechanobioreactor (Cellscale, Canada) at 0-20% strain, 1Hz for 4h/day.

RESULTS: Stepwise induction successfully generated SCL in 6 days as observed by drastically high PAX1 and NKX3.2, comparably very low non-SCL specific PAX3 **(Fig. 1B)**. Differentiation yielded an 8-fold increase in SCL relative to the starting population **(Fig. 1C)** and characteristic SCL morphology was observed **(Fig. 1D)**. The scRNAseq further confirmed the transition from iPSC-specific to SCL-specific transcriptional profiles, such as FOXC2, TWIST1 and SOX9 localizing in SCL clusters while loss of NANOG expression **(Fig. 1E)**. Reseeded SCL were induced toward SYN **(Fig. 2A)**, with IF confirming TNMD and MKX expression in SYN **(Fig. 2B)**. Subsequently, iTenocyte lineage under GDF5/CTGF treatment was established with an increase in MKX and TNMD **(Fig. 2C)**. Notably, both lineage commitment and proliferation, yielding up to a 200-fold expansion from the initial iPSC stage **(Fig. 2D)**. The YODA1 treatment at 1μM upregulated early tenogenic markers MKX and TNMD, while maintaining IL-1β levels comparable to dynamic mechanical stimulation in iTenocytes at physiological levels (5-10%, **Fig. 2E)**. Ongoing studies are evaluating the immunogenicity of iTenocytes in immunocompromised and immunocompent rats.

DISCUSSION: For the first time, scRNAseq revealed a clean separation of lineages at SCL stage, demonstrating our robust and optimized procedure for deriving tenogenic progenitor. Following SCL, SYN induction and subsequent iTenocyte generation were achieved by synergistic induction via GDF5/CTGF. Moreover, we identified YODA1 as a possible agonist in tenogenic ECM maturation to be used in iTenocyte induction.

SIGNIFICANCE: Off-target free, scalable and robust iTenocyte production may amend the clinical need for regenerative cell therapy in tendon-related injuries. This study is the proof-of-concept that development informed pathways can be combined with pharmacological stimulation to achieve reproducible induction of iTenocytes that may revolutionize our arsenal in the achieving scarless adult tendon regeneration.

ACKNOWLEDGEMENTS: This work was supported by California Institute for Regenerative Medicine (DISC0-14350 to DS and EDUC4-12751 to AEP).

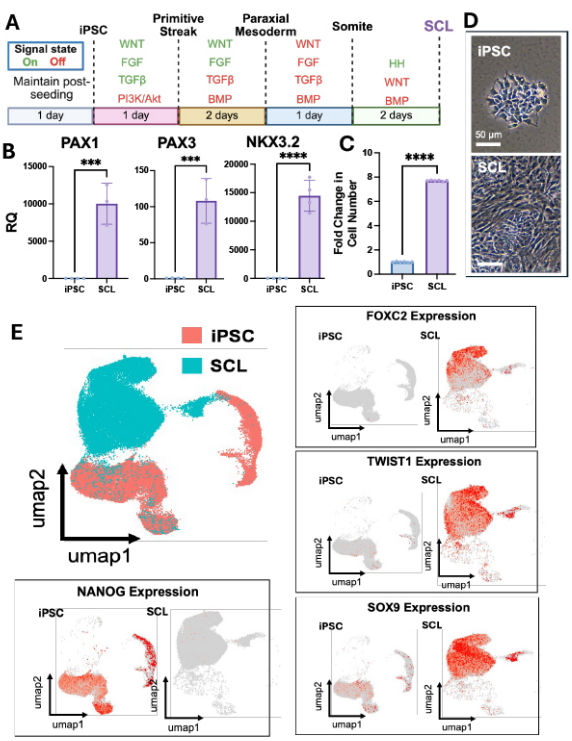

Figure 1. A) iPSC to SCL induction process within 6 days. **B)** Key SCL markers were detected successfully. **C)** There was 8-fold increase in cell numbers during SCL induction. **D)** SCL cells demonstrated distinct morphology compared to iPSCs. **E)** ScRNAseq analysis revealed a clear shift in cell types from iPSC to SCL. Detailed evaluation of NANOG, showed the loss of pluripotency, while SCL markers FOXC2, TWIST1 and SOX9 exclusive expression in SCL. Statical differences: ***p<0.001, ****p<0.0001.

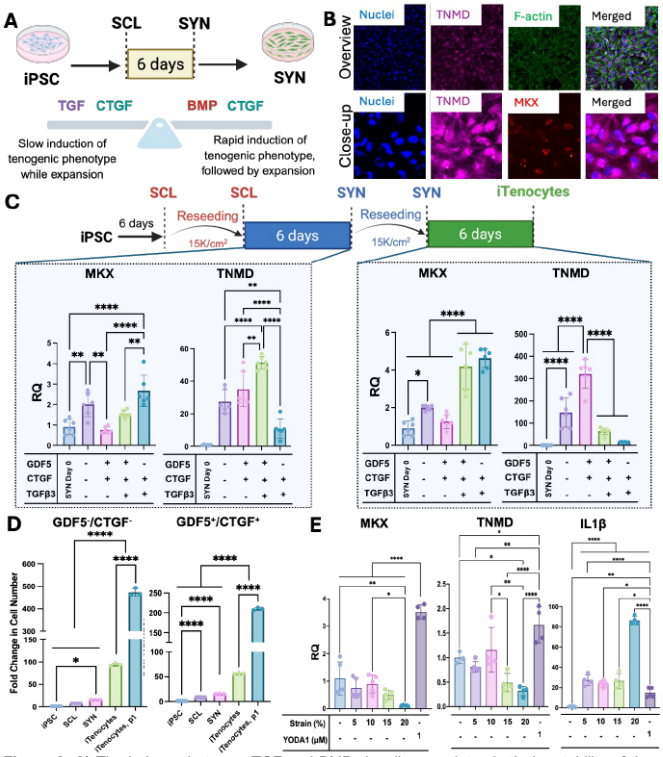

Figure 2. A) The balance between TGF and BMP signaling regulates both the stability of the tenogenic phenotype and the process scalability. **B)** GDF5 and CTGF promoted tenogenic differentiation, as confirmed by MKX and TNMD protein expression via immunofluorescence. **C)** Different combinations of BMP and TGF agonists were tested and GDF5/CTGF promoted stable tenogenic lineage commitment. **D)** YODA1 treatment elicited a stronger tenogenic response than dynamic mechanical stimulation in iTenocytes. Statical differences: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.