Aggrecan siRNA Treatment Improves Collagen Fiber Diameter in Tissue Engineered Meniscus

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INTRODUCTION: In early stages of meniscus development, proteoglycan content is low, and proteoglycan content increases with time.[1] *In vitro*, fibrochondrocytes (FCCs) produce GAGs early in culture, which can have inhibitory effects on collagen fiber formation during the maturation of tissue engineered (TE) meniscus.[1,2] This early over-production of GAGs, specifically those found in large aggregating proteoglycans (~100 GAG chains) such as aggrecan, inhibits the formation of aligned fibers and fiber bundles in TE menisci. Degradation of GAGs in early stages of development improves fiber formation; however, this non-specific degradation of all chondroitin sulfate GAGs from proteoglycans may be limiting the potential for further extracellular matrix (ECM) development by proteoglycans associated with collagen fibrillogenesis, such as small leucine rich proteoglycans (SLRPs).[3] Reincorporation of these GAGs after sufficient fiber development has been achieved would be necessary to ensure a robust TE construct. Small interfering RNA (siRNA) therapies specifically and temporarily inhibit the production of aggrecan without affecting other critical ECM components and without long-term inhibition of beneficial GAG deposition. The use of siRNA therapies to target and orchestrate matrix production for TE applications has not been widely studied. Therefore, the objectives of this study were to assess the effects of siRNA-induced inhibition of GAG production on the development of collagen fibers in tissue engineered meniscus constructs.

METHODS: Fibrochondrocytes (FCCs) were harvested from juvenile bovine menisci, as described previously.[4,5] siRNA/Lipofectamine complex was prepared by diluting siRNA and Lipofectamine RNAiMax into 4500 mg/L glucose DMEM. Components were combined and incubated with FCCs, which were centrifuged to form a pellet. ACAN siRNA was used as the aggrecan target siRNA, siGLO Red, Lipofectamine, and untransfected FCCs were used as controls. Linear meniscal constructs were made with transfected FCCs at a final concentration of $25x10^6$ cells/ml and 20 mg/ml collagen. Constructs were cultured in complete DMEM media with 4500 mg/L glucose at 37°C and 5% CO_2. Constructs were cultured for 30 days and media was changed and collected every 3 days. Constructs were either used for imaging or mechanically tested. Scanning electron microscopy (SEM) was performed to assess collagen organization. Fiber diameter and alignment were quantified using ImageJ and a custom Matlab code.[4,5] Tensile properties were determined using an ElectroForce 5500 System and pulled to failure at a rate of 1.5 mm/s for quasi-static loading.

RESULTS: ACAN siRNA treatment inhibited aggrecan production in 3D culture without affecting the production of collagen, demonstrating the feasibility of this tool for regulation of ECM production in TE meniscus constructs.[6] Therefore, we used this method to specifically inhibit aggrecan production without affecting other biosynthetic activity. SEM images show increased fiber bundling in ACAN transfected constructs, especially in regions surrounding lacunae. When quantified, these SEM images showed a >2 fold increase in the fiber diameter between the three control groups and the siACAN transfected group. siACAN transfected constructs showed a 117% ($p = 0.46$) increase in UTS, resilience showed a 90% ($p=0.16$) increase, and Young's Modulus showed a 125% ($p=0.37$) increase compared to control.

DISCUSSION: This study used ACAN siRNA to specifically control aggrecan production and manipulate fiber structure in TE constructs. The specific inhibition of aggrecan production using siRNA keeps SLRPs intact while removing the primary source of GAGs in the tissue, allowing for SLRPs to further facilitate matrix organization. Specific inhibition of aggrecan production produced a striking difference in fiber formation, specifically in fiber diameter, where we saw more than a doubling of fiber diameter in siACAN treated constructs compared to controls. This is similar to other studies where loss of aggrecan led to enhanced surface fibrillation in cartilage.[7] Additional work in an in vitro collagen gel showed that the presence of large aggregating proteoglycans decreased collagen fiber diameter in comparison to collagen alone.[8] Distinct architectural and functional changes are occurring in constructs with suppressed aggrecan production and these data support that a transient decrease in aggrecan promotes the formation of a more robust fiber network, especially larger fiber diameters, and trend to increase mechanical properties.

SIGNIFICANCE: These results show that siRNA therapies have potential to be used to specifically and temporarily inhibit production of proteoglycans by FCCs. Specific inhibition of aggrecan production improved collagen fiber diameter, producing fibers close to native fibers. Further exploration of proteoglycan content in developing TE meniscus constructs is needed to optimize collagen fiber formation.

REFERENCES: 1. Han W + 2016 2. Lintz M + 2022 3. Lopez S + 2023 4. McCorry MC + 2017 5. Ballyns JJ + 2010 6. Lopez S + ORS 2023 7. Li Q + 2020 8. Vogel K + 1989

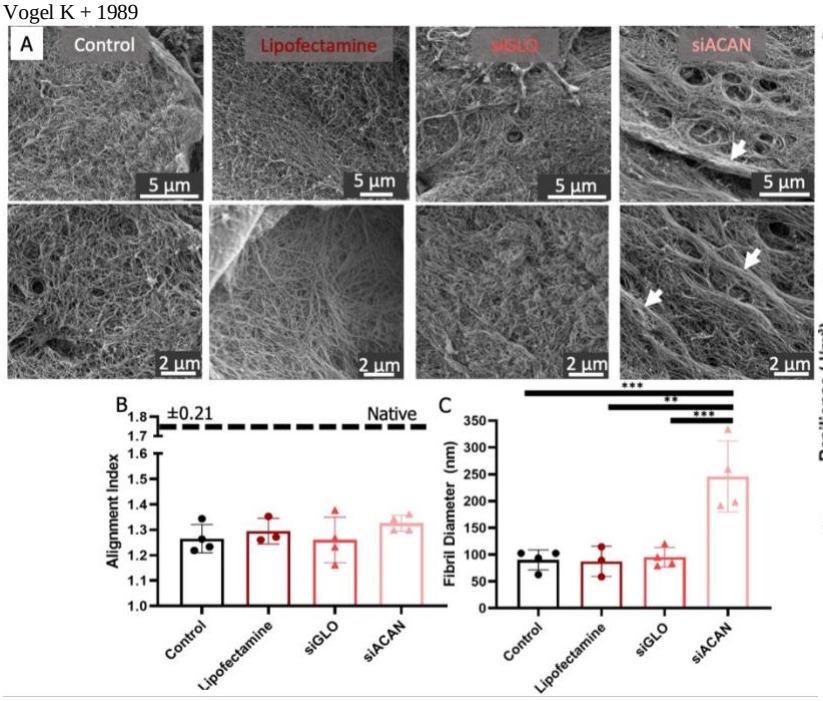

Figure 1: A) Representative SEM images of constructs after 30 days in culture. White arrows indicate large fibers. B and C) Quantitative analysis of fiber alignment and diameter in meniscus constructs. Analyzed using 1-way ANOVA with Tukey's multiple comparisons test (*=p<0.05)

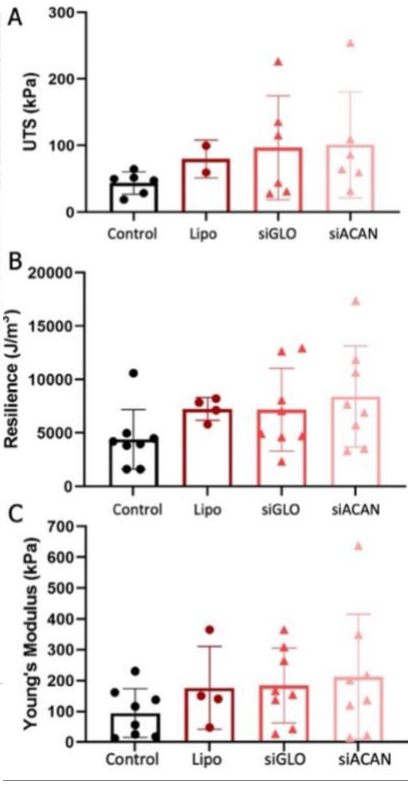

Figure 2: Tensile properties of meniscal enthesis constructs. Statistical error bars on graphs are ± standard deviation. Analyzed using 1-way ANOVA with Tukey's multiple comparisons test (*=p< 0.05).