# Investigating the Roles of microRNA-21 and microRNA-29 in Fibrosis

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INTRODUCTION: Arthrofibrosis is a common complication of total knee arthroplasty (TKA) and anterior cruciate ligament (ACL) injuries, where unresolved inflammation causes the buildup of scar tissue, leading to a loss in range of motion and reduction in quality of life. Unfortunately, there is no clinically effective treatment or prevention for arthrofibrosis, other than physical therapy or surgical removal of the fibrotic tissue. We aim to address the need to reduce the incidence of post-surgical arthrofibrosis by validating therapeutic microRNAs for reducing fibrosis. However, to do so, we need to determine the underpinnings of the role of microRNAs in fibrosis and determine candidate miRNAs. Here, we focus on the miRNA-29 family and miRNA-21 in an in vitro model of fibrosis, where the miRNA-29 family and the antagonist to miRNA-21 are predicted to act in an antifibrotic manner. We performed in vitro studies to examine the role of SMAD3 (mothers against decapentaplegic homolog 3) in fibrosis via transforming growth factor beta 1 (TGF-β1) and its regulation by miRNA-21 (profibrotic) and the miRNA-29 family (antifibrotic).

METHODS: Rat kidney fibroblasts (NRK-49F) were cultured in DMEM with 4.5 g/L D-glucose, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 ng/mL ascorbic acid. At 80% confluence, cells were treated with 0, 1, or 10 ng/mL TGF-β1 for 48 hours. Cell layers were lysed in radioimmunoprecipitation buffer, sonicated to disrupt the matrix, and the lysates were probed via blot for collagen I, collagen III, fibronectin, laminin, and vimentin. In separate experiments, NRK-49F cells at 80% confluence were treated for 48 hours with or without 10 ng/mL TGF-β1 and 0, 1, or 10 µM SMAD3 inhibitor SIS3 and were probed as above. NRK49Fs were also treated with 0, 1, or 10 ng/mL TGF-β1 for 48 hours before being harvested in Trizol®, followed by RNA extraction from the cell layer lysates by chloroform phase separation, isopropanol precipitation, and ethanol washes. The expression levels of miRNA-21, miRNA-29a, miRNA-29b, or miRNA-29c were examined by RT-qPCR. Statistics for all experiments were analyzed using one- or two-way ANOVA, followed by Tukey's post hoc testing between groups with a significance level of 0.05. Experiments were performed with a sample size of six cultures per variable and repeated to ensure validity.

RESULTS: After treatment with 1 ng/mL TGF-β1 for 48 hours, there was no change in collagen I, fibronectin, laminin, or vimentin compared to the no-treatment control, but collagen III was significantly increased, while treatment with 10 ng/mL TGF-β1 for 48 hours significantly increased the relative amounts of collagen I, collagen III, fibronectin, laminin, and vimentin (Figure 1). Treatment with 0 and 1 µM SIS3 did not affect the increase in the protein markers of interest when treated with 10 ng/mL TGF-β1. However, the group treated with both 10 µM SIS3 and 10 ng/mL TGF-β1 was significantly lower in all markers compared to the TGF-β1-only group and not significantly different from the no-treatment control. We also observed that TGF-β1 caused a significant increase in levels of miRNA-21 at 10 ng/mL, while both 1 and 10 ng/mL TGF-β1 caused a significant increase in the amount of miRNA-29a and miRNA-29b. No significant difference was seen at any level of TGF-β1 for miRNA-29c compared to the no-treatment control, relative to housekeeping miRNA-16 (Figure 2).

DISCUSSION: These results indicate that NRK-49F cells treated for 48 hours with 10 ng/mL TGF-β1 is a useful in vitro model of fibrosis. The effect of TGF-β1 was inhibited by SIS3, demonstrating that it is mediated by the canonical fibrosis pathway via SMAD3. TGF-β1 treatment also affects levels of the miRNA-29 family miRNA, decreasing expression relative to miRNA-16 at as low as 1 ng/mL TGF-β1. In contrast, miRNA-21 was significantly increased at 10 ng/mL TGF-β1. This indicates that these miRNAs are either directly involved in or directly impacted by fibrosis. These observations suggest that there is potential for miR-21 inhibitor and miR-29 mimic to act as therapeutics for preventing fibrosis. We plan to investigate this potential via transfection of the miRNA-29b mimic and miRNA-21 inhibitor.

SIGNIFICANCE/CLINICAL RELEVANCE: The results of the present study indicate a role for miR-21 and miR-29 in arthrofibrosis and the potential for miR-21 inhibitor and miR-29 mimic to act as therapeutics for preventing arthrofibrosis during total knee arthroplasty and anterior cruciate ligament injuries.

IMAGES AND TABLES:

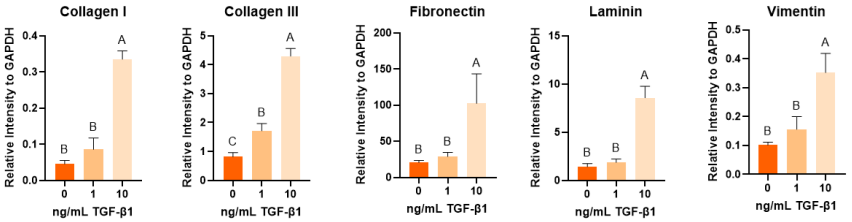


Figure 1: In vitro fibrosis model in NRK49F cells; NRK49F cells treated with 0, 1, or 10 ng/mL TGF-β1 for 48 hours and analyzed via western blot for collagen I, collagen III, fibronectin, laminin, and vimentin relative to GAPDH (n=6). Statistics were analyzed using one-way ANOVA, followed by Tukey's post hoc testing among all groups with a significance level of 0.05.

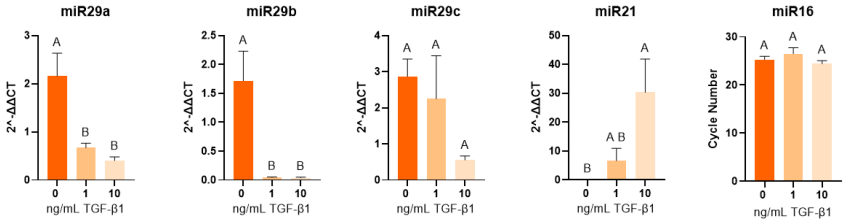


Figure 2: NRK49F cells treated with 0, 1, or 10 ng/mL TGF-β1 for 48 hours and evaluated for miR-29a, miR-29b, miR-29c, and miR-21 relative to miR-16 probed via stem-loop RT-qPCR. Statistics were analyzed using one-way ANOVA, followed by Tukey's post hoc testing among all groups with a significance level of 0.05.