Potential of forskolin in enhancing microfracture-based cartilage regeneration

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DISCLOSURES: The authors have nothing to disclose.

INTRODUCTION: The microfracture procedure is an arthroscopic, minimally invasive surgery for repairing osteochondral defects in the knees. During the surgery, small fractures are created in the surface of the subchondral bone. This minor fracturing stimulates mesenchymal stem cells (MSCs) from bone marrow to repair the defect. These cells will form a clot and then differentiate into chondrocytes during healing.[1] Unfortunately, there are inherent problems with the microfracture procedure. The native cartilage on the articular surface of the bones of the knee joint is normally composed of hyaline cartilage, which is primarily type II collagen. After the procedure, the new cartilage that is formed to repair the osteochondral defect is fibrocartilage. This cartilage is significantly more fibrotic than the native cartilage and is predominantly composed of type I collagen. Due to its structural properties, it also is more easily damaged and it degenerates more rapidly than hyaline cartilage. As a result, the fibrocartilage formed after this procedure tends to fail within about 3-5 years of the surgery.[2] In order to reduce the amount of fibrocartilage produced, we have studied fibrosis that occurs during new cartilage formation. Our recent work indicates that Yes-associated protein/transcriptional coactivator with PDZ binding motif (YAP/TAZ) expression in the Hippo pathway may be a therapeutic target. Previous studies have demonstrated that inhibition of YAP/TAZ reduces fibrosis.[3] There are specific drug candidates that have YAP/TAZ inhibiting potential, such as forskolin. Forskolin can act as an indirect YAP inhibitor. It does so by increasing cAMP that activates upstream large tumor suppressor 1/2 (Lats1/2) kinase activity, which subsequently increases phosphorylated YAP. Once YAP is phosphorylated, it will not translocate to the nucleus and be expressed, thereby reducing fibrosis proliferation.[4,5]

We seek to optimize cartilage repair and generation after microfracture surgery by utilizing YAP inhibitors. We first used an *in vitro* chondrogenesis model to test the influence of YAP inhibitors, specifically forskolin in this study, on new cartilage formation. Next, based on the conditions optimized *in vitro*, we will introduce forskolin post-microfracture and test its capacity to promote hyaline cartilage formation *in vivo*. We further hypothesize that the administration of forskolin or other YAP inhibitors will reduce fibrosis and increase hyaline cartilage formation, thus improving the longevity of microfracture surgery.

METHODS: With IRB approval, human MSCs were isolated from bone marrow samples. To reach sufficient cell numbers, MSCs from multiple donors were pooled and expanded in growth media. 1 x 10^6 P6 MSCs were subsequently encased into a 44 µL fibrin scaffold to mimic the formation of a blood clot after the microfracture procedure. The MSCs were differentiated into chondrocytes within the fibrin scaffold over the course of 28 days in chondrogenic media. At 0, 7, or 14 days of chondrogenesis, forskolin was introduced into the culture with concentrations of 1 µM or 10 µM to simulate their potential influence on new cartilage formation post microfracture. Treated chondrocytes were analyzed by RT-qPCR and one-way ANOVA test with Tukey's post-hoc test, western blot, and histology to assess the extent of fibrosis and cartilage formation.

RESULTS: In all three tested time points, forskolin at both doses could suppress the expression of representative markers of fibrosis, including type 1 collagen (*COL1*) and type 3 collagen (*COL3*), in MSC-derived cartilage, as indicated by the results from RT-qPCR analysis in **Figure 1**. However, a significant difference between the control and treatment groups was only observed when the dose was at 10µM for *COL1* ($p < 0.0001$) at all time points. To further validate the findings from RT-qPCR, western blot was performed to examine the protein levels of *COL1*. As shown in **Figure 2**, at 10 µM, forskolin significantly reduced *COL1* levels, suggesting its potential to inhibit fibrosis in the chondrogenesis of MSCs. The highest degree of *COL1* suppression was observed when forskolin was introduced at day 7 of chondrogenesis.

DISCUSSION: Our results from in vitro study indicate that the administration of forskolin reduces *COL1* and *COL3*. It is also apparent that 10 µM of forskolin was a sufficient dose to reduce fibrosis markers at multiple time points. There was also no apparent impairment in chondrogenesis based on Safranin-O staining. This indicates that targeting the YAP/TAZ pathway is a potential therapeutic option. Of note, forskolin is a relatively nonspecific inhibitor of this pathway as it increases cAMP and, therefore, causes downstream phosphorylation of YAP/TAZ, so we seek to further test this with our experiments utilizing other more specific YAP inhibitors. We recognize this *in vitro* model cannot capture the complexities of the knee joint after a microfracture procedure. There may be varying degrees of cartilage injury and physiologic conditions that may vary from the model we are utilizing. In the future, we will test the potential of introducing forskolin and other YAP inhibitors to enhance microfracture-based cartilage regeneration in rats.

SIGNIFICANCE/CLINICAL RELEVANCE: The microfracture procedure currently lacks durability and can fail in a relatively short time period so enhancing this surgical technique with an interarticular injection of an anti-fibrotic agent may help reduce fibrosis formation and improve longevity. We ultimately hope to improve the integrity of repaired cartilage and reduce the progression of osteoarthritis due to chondral injury.

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IMAGES AND TABLES:

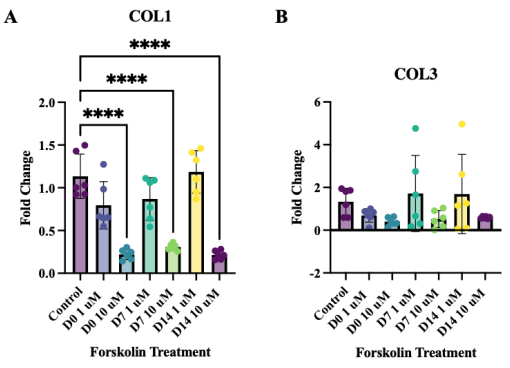

Figure 1. A. *COL1* and B. *COL3* gene expression in MSC-derived cartilage after being treated with 1 µM or 10 µM of forskolin at day 0, day 7, or day 14 of chondrogenesis. ****, $p < 0.0001$.

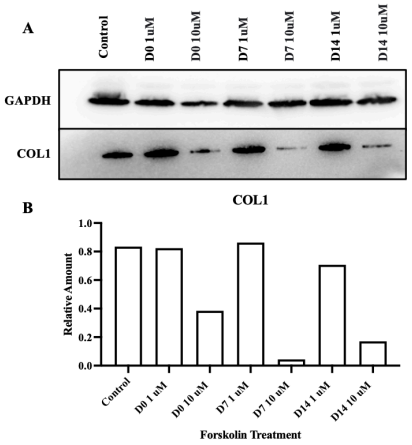

Figure 2. A. *COL1* protein levels in in MSC-derived cartilage after being treated with 1 µM or 10 µM of forskolin at day 0, day 7, or day 14 of chondrogenesis. B. The relevant protein levels.