Inhibition of Cellular Contractility Synergistically Improves TGF-β3-mediated Chondrogenesis

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Introduction: Cartilage injury is one of the most prevalent connective tissue injuries and results in pain and reduced mobility. Given the limited regenerative capacity of cartilage, it is unable to self-heal. The use of marrow-derived stem cells, whether recruited via marrow stimulation or isolated for tissue engineering approaches, is a promising avenue of cartilage regeneration research. However, without the use of additional influences, such as growth factors and pharmacological agents, marrow-derived cells are predisposed to deposit fibrocartilaginous tissue[1,2]. This fibrous tissue can provide short term symptomatic relief but does not fully replicate the original hyaline cartilage found in joints. The limited success of marrow-derived cells alone implicates a need for better chondrogenic therapies that can augment the use of marrow cells by inducing improved chondrogenic differentiation. However, the microscale properties of the cells involved, particularly cellular contractility and mechanics, have not been thoroughly investigated. One potential area of interest is the Rho ROCK pathway, a mediator of cell contraction, adhesion, and shape. Previous studies have shown that inhibition of the Rho ROCK pathway alters fibroblast phenotype by regulating actin organization and the expression of SOX9, a chondrogenic transcription factor[3,4]. The purpose of this study was to evaluate how Rho ROCK inhibition facilitates TGF-β3-induced chondrogenic differentiation of bone marrow-derived cells.

Methods: To model the cells recruited to cartilage defects during marrow stimulation, bone marrow-derived cells (MDCs) were isolated from juvenile bovine femoral condyles and utilized for all studies (P1-P2). Cell Mechanics: To evaluate the influence of Fasudil and TGF-β3 on cellular mechanical properties, MDCs were cultured in basal media (10% FBS, 1% PSF) in 6-well plates for three days, after which the media was replaced with basal media containing Fasudil (Fas; Rho-ROCK inhibitor; 50μM) and/or TGF-β3 (10ng/mL) for 45 minutes. Individual cell mechanics were measured using image-guided nanoindentation (Optics 11 Pavone; 3μm radius probe), and load-deformation curves were used to obtain Effective Young's modulus. SMAD2/3 Nuclear Activity: To assess the impact of inhibiting Rho ROCK on nuclear SMAD2/3 activity, MDCs were cultured in basal media in an 8-well chamber slide for three days. The media was then replaced with chemically defined media (CDM) or CDM containing Fasudil for 24 hours. TGF-β3 (10ng/mL) was then applied for 60 minutes, and cells were fixed and stained for SMAD2/3 (TGF-β3 activation) and phalloidin (F-Actin visualization). Cells were imaged using confocal microscopy (Nikon A1R) and analyzed for nuclear SMAD2/3 intensity. 3D Fibrin Gel Studies: To evaluate the effect of Rho ROCK inhibition on TGF-β3-mediated chondrogenesis, we utilized a fibrin gel system (50mg/mL fibrinogen, 10U/mL thrombin, 2M/mL MDCs). Gels were cultured in basal media or media containing Fasudil and/or TGF-β3 for four weeks. Gels were imaged three times per week and at the final time point to determine contraction over time. After the culture period, the constructs were harvested for gene expression of chondrogenic markers.

Results: Nanoindentation studies showed reduced effective Young's modulus in Rho ROCK-inhibited cells, an effect which was maintained with the addition of TGF-β3 (**Fig. 1A**). Also, actin stress fiber formation in Rho-ROCK inhibited cells was attenuated compared to controls (**Fig. 1B**). Furthermore, there was a clear activation of SMAD2/3 signal in the nucleus with TGF-β3 treatment; interestingly, we observed a stepwise trend in nuclear intensity of SMAD2/3 in cells treated with Fasudil, TGF-β3, and Fasudil with TGF-β3 (**Fig. 2A & 2B**). Finally, for 3D gels, Fasudil mitigated gel contraction compared to control in both basal media and media containing TGF-β3 (**Fig. 3A**). A 9-fold increase in type II collagen expression was observed in gels cultured with Fasudil alone, and ~200-fold increase in gels cultured with Fasudil and TGF-β3 (**Fig. 3B**).

Discussion: The results of this study show that Rho ROCK inhibition with Fasudil had a significant impact on cell behavior and mechanics, particularly when applied in addition to TGF-β3. Fasudil treatment decreased cellular stiffness and stress fiber formation, even in cells co-treated with TGF-β3, potentially having implications on chondrogenic fate. When treated with both Fasudil and TGF-β3, cells exhibited an increased response to TGF-β3 as demonstrated by enhanced SMAD 2/3 nuclear intensity after a short period of time, indicating that Rho-ROCK inhibition improves early TGF-β3 activation of the SMAD 2/3 pathway. Macroscale studies showed that Fasudil and TGF-β3 treatment significantly enhances type-II collagen deposition over four weeks, while simultaneously reducing contraction. This combined effect suggests that Fasudil treatment would help maintain defect fill while simultaneously promoting cartilage regeneration. Exploring the interplay between the Rho ROCK pathway and TGF-β3 could potentially provide a deeper insight into strategies for fostering chondrogenesis following marrow stimulation while maintaining the volume of the repair tissue.

Significance/Clinical Relevance: Investigating the roles of the Rho ROCK pathway and TGF-β3 in the early cartilage repair environment offers valuable insights into potential pharmacological strategies for enhancing marrow stimulation.

References: [1] Freemont+, Eur J Radiol, 2006. [2] Richardson+, Methods, 2016. [3] Gill+, Am J Physiol Cell Physiol, 2008. [4] Woods+, J Biol Chem, 2005.

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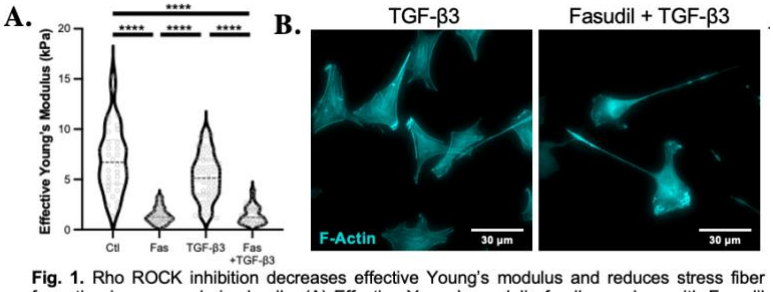

Fig. 1. Rho ROCK inhibition decreases effective Young's modulus and reduces stress fiber formation in marrow-derived cells. (A) Effective Young's moduli of cells on glass with Fasudil (50uM) and TGF-β3 (1ng/mL) for 45 minutes. (B) Actin morphology of cells treated with Fasudil (50uM) and TGF-β3 (1ng/mL) for 45 minutes and cultured on glass in basal media.

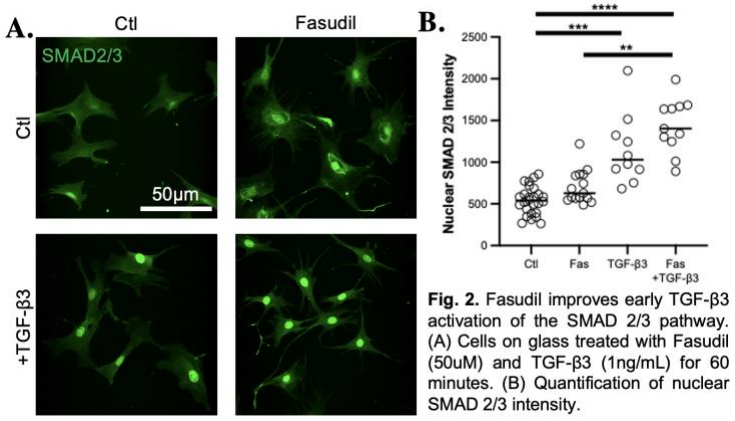

Fig. 2. Fasudil improves early TGF-β3 activation of the SMAD 2/3 pathway. (A) Cells on glass treated with Fasudil (50uM) and TGF-β3 (1ng/mL) for 60 minutes. (B) Quantification of nuclear SMAD 2/3 intensity.

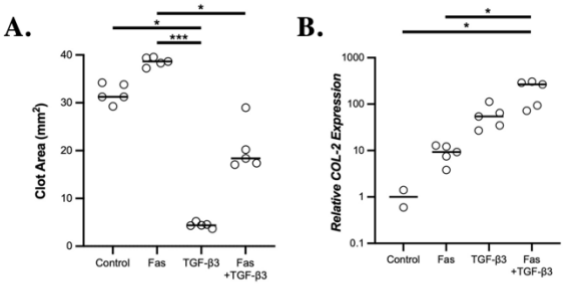

Fig. 3. Clot area decreases and COL II expression increases with Fasudil treatment. (A) Final area of fibrin clots after four weeks in culture in Fasudil (50uM) and TGF-β3 (1ng/mL) media. (B) Gene expression of COL II within fibrin clots at the terminal timepoint.