Directing the Chondro-Fibro Axis with Cellular Contractility for Volumetric and Functional Cartilage Repair

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INTRODUCTION: Microfracture (MFx), the gold standard of cartilage repair, exhibits poor long-term survivorship, often due to inadequate defect fill and inferior fibrous scar formation [1,2]. The early MFx clot is relatively unexplored, even though its early contraction and fibrotic susceptibility hinder the cartilage regeneration potential. To combat these issues, the objective of this study was to combine Rho-ROCK inhibition (via Fasudil) with anabolic TGF-β3 stimulus [3] to promote specific chondrogenesis over fibrosis ("chondro-fibro axis"; Fig 1A) for volumetric and functional cartilage repair.

METHODS: *In Vitro* – To simulate MFx clots, we encapsulated bovine marrow-derived cells (MDCs; nonsorted; male only due to availability from slaughter) within fibrin microgels (10μL), which were cultured for 3 days with TGF-β3 (10ng/mL). Subsets of gels were cultured with either Fasudil (ROCK inhibitor, 10μM) or LPA (Rho activator, 10μM). Constructs were stained for SOX9 and α-SMA as early markers of MDC chondrogenesis and fibrosis (Fig. 1B), respectively. Additional labeled fibrin gels were cultured for one week in GAG-labeling medium (azide-modified galactosamine) for visualization of nascent deposition. Fibrin macrogels with MDCs (100μL) were cultured for four weeks (+/- TGF-β3, +/- Fasudil within the initial gel) to track contraction, matrix deposition, and gene expression. *In Vivo* –TGF-β3+Fasudil was evaluated in full-thickness trochlear defects (2mm diameter) in male rats. Following MFx [4], a solution containing thrombin and calcium chloride was injected +/- TGF-β3 and +/- Fasudil for incorporation into clotted fibrin. At 8-weeks, repair tissue and neighboring cartilage mechanics were evaluated, and Alcian Blue/Nuclear Fast Red staining was performed on sections.

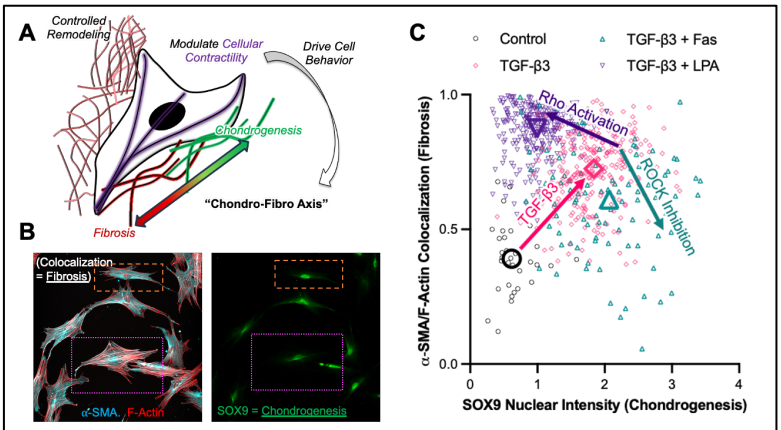

Figure 1. Chondro-Fibro Axis. [A] Schematic of cellular contractility driving the axis between chondrogenesis and fibrosis. [B] MDCs stained for α-SMA and F-actin, with colocalization representing early fibrosis, and SOX9 staining as a marker of chondrogenesis. [C] α-SMA/F-actin colocalization versus SOX9 nuclear intensity as metric of chondro-fibro axis, with control, TGF-β3, LPA+ TGF-β3 (Rho activation), Fasudil+ TGF-β3 (ROCK inhibition). N>30 cells/group.

RESULTS: TGF-β3 treatment alone increased both SOX9 and α-SMA activity, with little change along the chondro-fibro axis. LPA-TGF-β3 treatment decreased SOX9 activity and increased α-SMA staining, while. Fasudil-TGF-β3 treatment increased the ratio between SOX9 and α-SMA (Fig. 1C). In the first week of culture, TGF-β led to significant fibrin remodeling, which was almost completely mitigated with Fasudil incorporation. Furthermore, while reduced, these cells still demonstrated an initial ability to deposit GAG (Fig. 2A). Macroscopically, Fasudil treatment completely mitigated TGF-β3 driven fibrin gel contraction (Fig. 2B), and Fasudil+TGF-β3 co-treatment led to robust proteoglycan and type II collagen deposition (Fig. 2C). Finally, we observed considerable increases in both aggrecan gene expression, as well as an increase in COL2:COL1 gene expression, giving promise to co-treatment in preferentially increasing chondrogenesis while maintaining volume. In vivo, TGF-β3 +/- Fasudil treatment was successfully incorporated into a MFx clot (Fig. 3A/B). At 8-weeks, while TGF-β3+Fasudil treatment did not improve repair tissue mechanics (Fig. 3C), it provided the best protection of the neighboring cartilage (Fig. 3D). Initial histology demonstrated a deeper blue stain in combination treatment defects (Fig. 3E)

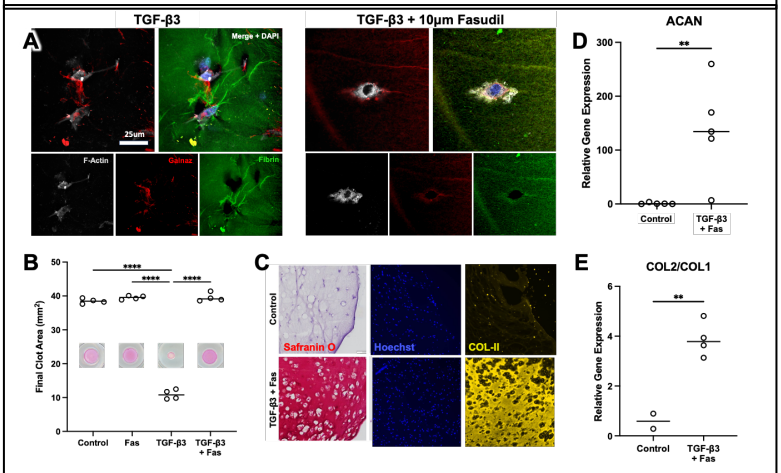

Figure 2. Combination TGF-β3-Fasudil Treatment. [A] MDCs cultured within fibrin gels (green) for one week. Metabolic labeling for nascent GAG (Galnaz; red) and cell staining with phalloidin (white). TGF-β3 and TGF-β3+Fasudil treatment. [B] Areas of MDC-fibrin gels, with embedded Fasudil and TGF-β3, cultured for 4 weeks. [C] Safranin O-Fast Green, Hoechst, and type 2 collagen (COL-II) staining of constructs at end of culture. [D] Aggrecan (ACAN) and [E] COL2/COL1 ratio gene expression of MDC-fibrin gels after 4-week culture. **, **** represent p<0.01, 0.0001.

DISCUSSION: MFx contraction and fibrosis, which are exacerbated by TGF-β3, limit repair efficacy and duration. Rho-ROCK modulation appears to direct MDC activity along this axis between chondrogenesis and fibrosis. While Fasudil may slightly reduce early GAG deposition, it mitigated TGF-β3 mediated contraction, and co-treatment led to considerable cartilage-specific matrix deposition. Finally, initial *in vivo* studies show promise towards this strategy, and future work will translate this approach to both female rats and a minipig model.

SIGNIFICANCE/CLINICAL RELEVANCE: Maintaining MFx fill while preferentially guiding chondrogenesis would represent a novel, impactful method to improve cartilage repair outcomes.

REFERENCES: [1] Erggelet+ 2016. [2] Martin+ 2019. [3] Wang+ 2015. [4] Chihab+ 2024.

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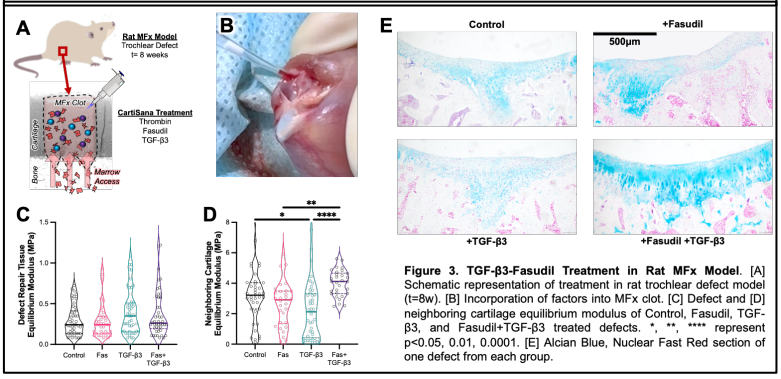

Figure 3. TGF-β3-Fasudil Treatment in Rat MFx Model. [A] Schematic representation of treatment in rat trochlear defect model (t=8w). [B] Incorporation of factors into MFx clot. [C] Defect and [D] neighboring cartilage equilibrium modulus of Control, Fasudil, TGF-β3, and Fasudil+TGF-β3 treated defects. *, **, **** represent p<0.05, 0.01, 0.0001. [E] Alcian Blue, Nuclear Fast Red section of one defect from each group.