

# A Small Molecule TGF- $\beta$ Agonist Drives Fibrous Tissue Formation in Meniscus Tissue after Injury

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**Introduction:** The meniscus, a crucial load-bearing fibrocartilaginous structure in the knee, is commonly injured and has a poor innate healing capacity despite surgical apposition of tear edges.<sup>1</sup> A contributing factor to this poor healing is the loss of matrix at the tear interface, resulting in a substantial tissue gapping.<sup>2</sup> To address this, numerous factors have been explored to promote cell migration and tissue deposition at this interface.<sup>3-5</sup> Namely, TGF- $\beta$ , a pleiotropic cytokine, can stimulate meniscal fibrochondrocytes (MFCs) matrix production and promotion of tissue integration both in vitro and in vivo. However, the cost and propensity for loss of biologic activity when incorporated into drug delivery systems make application of this molecule a challenge. A small molecule agonist of this pathway may be better suited for these applications. To that end, we evaluated whether the small molecule TGF- $\beta$  signaling agonist, SRI-011381 hydrochloride (referred to as SRI) could promote anabolic matrix production in MFCs. We first queried the structural and micromechanical changes local to vertical meniscus tears over 3 weeks following injury to identify ideal treatment windows. Next, we evaluated the ability of SRI to promote myofibroblastic conversion of MFCs in vitro on a stiff substrate. Using this information, we then tested whether the SRI could successfully promote anabolic gene expression of MFCs in fibrin, a softer 3D delivery vehicle commonly used in meniscus tear augmentation strategies.

**Methods:** Minipig Surgery Model: Juvenile (6-month-old) Yucatan minipigs underwent bilateral parapatellar surgery and each medial meniscus received a vertical longitudinal tear (1/2 arc length, red-white zone) (n=1 animal/time point) (Fig 1A). Animals were euthanized at 1, 2, or 3 weeks and menisci were harvested for a series of micro- and macro-scale analyses. Atomic Force Microscopy (AFM) Nanoindentation: Histology guided AFM (Fig 1B-C,D) was applied to 40  $\mu$ m-thick cryo-sections (n=2/group) in PBS using a microspherical tip ( $R \approx 6 \mu$ m, nominal  $k \approx 0.6$  N/m) and Dimension Icon AFM. For each region (defect edge and body), the effective indentation modulus was calculated<sup>6</sup> for 50-75 locations. 2D MFC Activation Assay: Bovine MFCs were cultured in chemically defined medium (CM) on glass slides and maintained in either 1) CM, 2) CM + TGF $\beta$  (10 ng/mL), or 3) CM + SRI (10  $\mu$ M) for 4 days. Vehicle control = DMSO. Cells were then fixed and stained for actin and  $\alpha$ SMA with a DAPI nuclear counterstain. Confocal z-stacks were obtained at 20x (n=10/group), and the  $\alpha$ SMA:actin correlation coefficient and cell area were measured using CellProfiler (Fig 2A-C). 3D MFC Activation Assay: MFCs (~50,000) were suspended in 75  $\mu$ L of fibrin adhesive (Tisseel<sup>TM</sup>) and cross-linked into cylindrical constructs (n=3/group) for 5 minutes (Fig 3A). Constructs were cultured for 14 days, maintained in either 1) CM, 2) CM + TGF $\beta$ -3 (10 ng/mL), or 3) CM + SRI (10  $\mu$ M). Constructs were rinsed with sterile PBS (3x) and processed for RNA extraction and cDNA synthesis followed by qRT-PCR for anabolic genes downstream of TGF- $\beta$ . Statistical Analyses: For AFM of meniscus defect edge and body, paired t-tests were used. 1-way ANOVA with repeated measures and Sidak's correction for multiple comparisons was used to evaluate  $\alpha$ SMA:actin correlation, cell area, and qPCR.

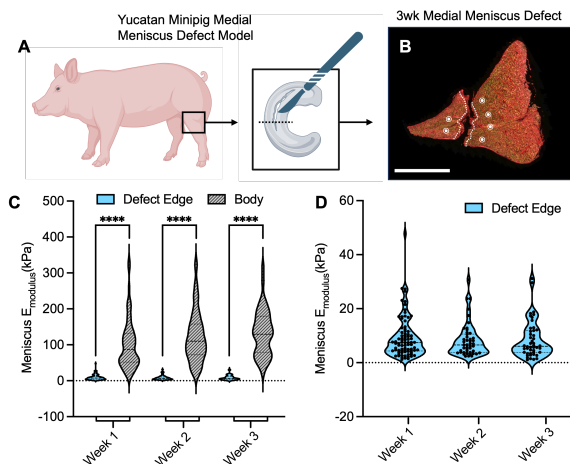
**Results:** The region adjacent to a meniscus tears had a decreased indentation modulus within 1-week post injury, compared to regions further removed from the injury site, with a similar trend seen at both 2- and 3-weeks (Fig 1A-D). MFCs cultured on a stiff substrate (glass) responded to SRI by increasing their  $\alpha$ SMA:actin correlation coefficients and cell area, reaching levels similar to that of TGF $\beta$ -3 treated MFCs (Fig 2A-C). In 3D culture, fibrin + MFCs supplemented with SRI showed increased expression of genes downstream of TGF- $\beta$ , including DCN, SOX9, YAP, COL1A1, and ACTB, reaching levels higher than constructs treated with TGF $\beta$ -3 (Fig 3A-B).

**Discussion:** Our findings show that the edges of meniscus defects decrease in stiffness within 1-week of injury. This rapid loss in matrix integrity soon after defect creation indicates that delivery of factors to promote matrix deposition and prevent local degradation would be best delivered soon after the injury has occurred. Notably, when treated with the TGF- $\beta$  agonist SRI in vitro, MFCs showed evidence of myofibroblastic conversion (higher  $\alpha$ SMA:actin correlation and cell area)<sup>7</sup>. This transition correlates with an increase in cell contractility and ECM deposition, necessary functions at the defect edge to promote tissue formation<sup>8</sup>. MFCs cultured in fibrin treated with SRI also showed increased expression of TGF- $\beta$  induced genes, even in this softer microenvironment. Taken together, these data indicate that SRI may be a promising small molecule to deliver to the site of meniscus injury to promote repair. Future work will evaluate the efficacy of SRI when delivered to meniscus tears in a large animal injury model.

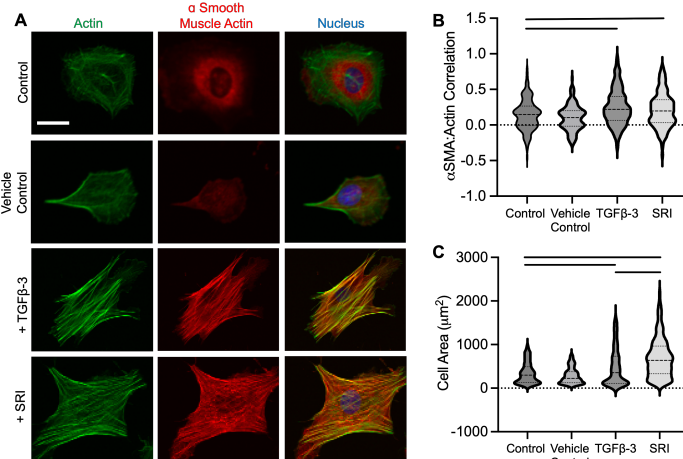
**Significance/Clinical Relevance:** We show that a novel small molecule agonist of TGF- $\beta$  signaling can promote anabolic MFC function in a clinically translatable fibrin adhesive. This may be used to improve outcomes by delivering a stable, efficacious small molecule directly to the meniscus repair site.

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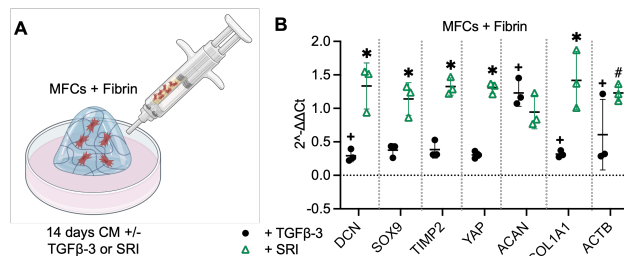
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**Fig 1.** (A) Schema of meniscus injury model (black line = orientation of sectioning) and (B) representative Picrosirius Red staining (10x, white line = defect edge and  $\odot$  = body indentation regions, scale bar = 1mm). AFM modulus of (C) defect edge vs. body and (D) defect edge for all timepoints. Mean  $\pm$  SD, \*\*\*\*indicates  $p < 0.001$ .



**Fig 2.** (A) Representative MFCs stained for actin and  $\alpha$ SMA after exposure to TGF- $\beta$ -3 or SRI. (B)  $\alpha$ SMA:actin correlation coefficient and (C) cell area. Mean  $\pm$  SD, bars indicate  $p < 0.05$ . Scale: 15 $\mu$ m.



**Fig 3.** (A) Schema of MFCs + fibrin construct fabrication. (B) qPCR results (dotted line indicates no treatment (CM) group). \* indicates ( $p < 0.05$ ) or # ( $p < 0.1$ ) vs. TGF- $\beta$ -3, + indicates difference ( $p < 0.05$ ) vs. CM alone.