Piezo1 Overaction Induces a Myofibroblast Phenotype in Synovial Fibroblasts via YAP

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INTRODUCTION: The mechanisms of emergence and contributions of myofibroblasts to post-traumatic osteoarthritis (PTOA) are not yet characterized. In other tissues, activation of mechanoreceptor Piezo1 via a stiffened microenvironment and subsequent translocation of Yap1 to the nucleus contributes to fibrosis [1,2,3] and overactivation of canonical Wnt/β-catenin signaling (cWnt) (highly relevant to synovial fibrosis) [4,5,6]. We recently proposed a potential role for Piezo1 in mediating myofibroblast emergence and demonstrated Piezo1 upregulation in PTOA synovium [7,8]. Herein, we sought to establish Yap1 as the mechanistic intermediary between Piezo1 signal transduction and the induction of the myofibroblast phenotype in synovial fibroblasts.

METHODS: Joint injury: Mice underwent noninvasive unilateral anterior cruciate ligament rupture (ACLR) [9,10]. Lineage tracing: Confocal microscopy of cryosections of *Acta2*-Cre^{ERT2};tdTom mice co-labeled with EdU were used to trace *Acta2*+ cells in uninjured and injured synovium (IP tamoxifen at -2, 0, and +2 days post-ACLR). Flow Cytometry quantified the number and proportion of *Acta2;*tdTom+ fibroblasts (CD45- CD31- CD146-). Synovial stiffness: AFM-nanoindentation was applied to sagittal cryo-sections of injured and uninjured limbs (20 indentations/limb). *In vitro* treatments: P3 pooled primary synovial fibroblasts (SFs) from 6 WT mice were treated with Veh (DMSO), Piezo1 agonist (10 μM Yoda1), Yap1 inhibitor (0.5 μM Verteporfin), or both Piezo1 agonist and Yap1 inhibitor. αSMA/F-actin ICC: 80% confluent SFs were immunostained for αSMA, F-actin, and DAPI following 1d of treatment. Average fluorescent intensity and overlap of αSMA and F-actin were evaluated in MATLAB to identify *bona fide* myofibroblasts. Scratch assay: SFs were raised to 90% confluency in 24 well plates before wounds were introduced with a p200 tip and imaged longitudinally. The proportion of initial wound site recovered by migrating SFs was quantified. Gel contraction assay: SFs were seeded into 3D collagen discs (~1 x 10^6 cells/gel) in 24 well plates with experimental media for 1d and then released from wells. Gel surface area was longitudinally quantified in ImageJ as a metric of contractility. Macrophage crosstalk: Conditioned SF media after treatment with +/- Yoda1 and +/- Verteporfin was added onto polarized macrophages before RNA was extracted and evaluated.

RESULTS: ACLR resulted in significant stiffening (75.6%) of synovial tissue (**Fig 1A**), concurrent with emergence of *Acta2;*tdTom+ and proliferative (EdU+) synovial cells (**Fig 1B-C**) in PTOA synovium. Piezo1 agonism significantly increased *Acta2, Yap1,* and cWnt target gene *Axin2* expression, implicating Piezo1 in myofibroblast phenotype through Yap1 and cWnt (**Fig 1D).** Piezo1 agonism enriched αSMA protein, which co-located to F-actin (~80% ± 6% overlap) (**Fig 1E**), confirming a myofibroblast phenotype. Piezo1 agonism also increased the pro-fibrotic functions of migratory wound closing (**Fig 1F**) and contractile activity (**Fig 1G**). Yap1 inhibition completely ablated these effects. Relevant to paracrine crosstalk between SFs and macrophages, conditioned medium from Piezo1 agonist-treated SFs elicited anti-inflammatory effects (↓Il1b, ↓Nos2, ↑IL10) but also upregulated *Tgfb1* in macrophages. Yap1 inhibition also ablated these effects, demonstrating that macrophage-activating secreted factors released after Piezo1 activation are also dependent upon Yap1 (**Fig 1H**).

DISCUSSION: We demonstrate Piezo1 activation is upstream of a myofibrotic SF phenotype via the critical Yap1 intermediary. While Piezo1 agonism enhanced myofibroblast activity at transcript, protein, and functional levels, Yap1 inhibition ablated this phenotype. These findings suggest that in PTOA, Yap1 may be required to direct Piezo1-mediated mechanobiological induction of pro-fibrotic myofibroblast-like SFs, which we are currently testing with *in vivo* gain- and loss-of-function studies focused on Piezo1 and Yap1. Our data confirm that, alongside well-characterized histological and molecular indicators of fibrosis in the ACLR model, synovial stiffening (75.6%) occurs, which may underpin Piezo1 overactivation. Lineage tracing using *Acta2;*tdTom reporter mice corroborated our prior scRNAseq-based description of myofibroblast emergence following joint injury but partially resolves by 28d post-ACLR [8].

SIGNIFICANCE/CLINICAL RELEVANCE: There is no effective treatment for PTOA, but an improved understanding of mechanotransduction mechanisms mediating a fibrotic and stiffened synovium will assist in drug development to intervene in onset of fibrosis and inflammation.

REFERENCES: [1]Fu+ 2021, [2]Bartoli+ 2022, [3]Swain+ 2022, [4]Zhou+ 2020, [5] Liu+ 2020, [6]Zhou+ 2017, [7]Bergman & Lammlin+ 2023, [8] Knights+ 2022, [9]Christiansen+ 2012, [10] Maerz+ 2015

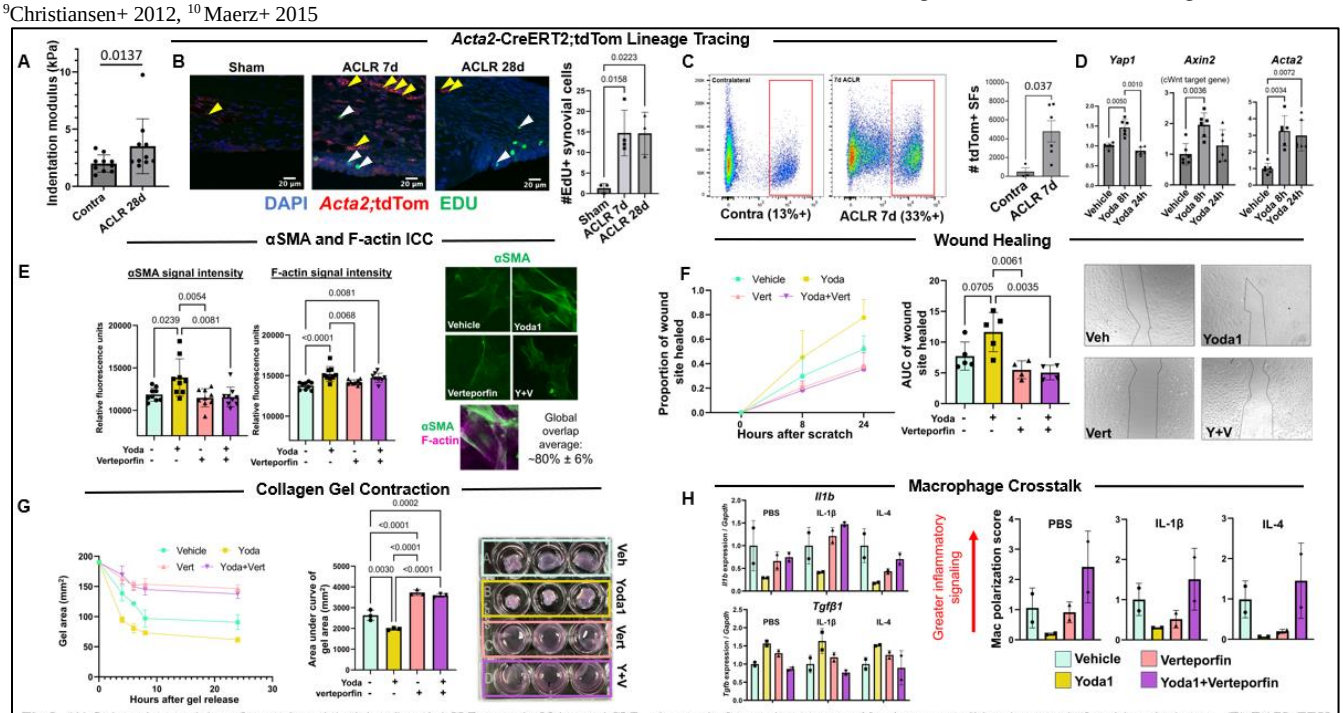

Fig 1. (A) Indentation modulus of contralateral (uninjured) and ACLR synovia 28d post-ACLR, via atomic force microscopy. n=10 mice per condition (even male/female), paired t-test. **(B)** DAPI, EDU (proliferative, white arrows), and *Acta2;*tdTom+ (yellow arrows) signal in Sham, 7d, ad 28d-post ACLR joints from *Acta2-CreERT2;*tdTom mice. Tamoxifen at -2, 0, +2 post-ACLR. **(C)** Flow cytometry plot (left) and quantification (right) of contralateral (uninjured) and ACLR synovium from *Acta2-CreERT2;*tdTom mice. n=4-8. Unpaired t-test. **(D)** Gene expression of *Yap1, Acta2* and *Axin2* after Veh or Yoda1 treatment, n=6 bio. replicates, ANOVA. **(E)** IF stain and quantification of αSMA filaments in SFs (n=9 rep., ANOVA). Phalloidin counterstain of F-actin to identify myofibroblasts. **(F)** Wound healing after scratch in SFs treated with Veh, Yoda1, Verteporfin, or Yoda1+Verteporfin and representative scratch sites after 1d (n=4-5 rep., ANOVA). **(G)** Quantification of collagen gel surface areas containing SFs treated with Veh, Yoda1, Verteporfin, or Yoda1+Verteporfin and final gel images after 1d of contraction (n=3 rep., ANOVA). **(H)** *Tgfβ1* and *Il1β* gene expression and macrophage polarization scores (ratio of *Il1β, Il6,* and *Nos2* over *Mrc1, Il10,* and *Arg1*) of Veh-, Il1β-, or Il4-polarized macrophages after treatment for 1d with conditioned media from SFs treated with Veh, Yoda1, Verteporfin, or Yoda1+Verteporfin.