A Novel Role for the MRTF/SRF Pathway in Tendon Development

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INTRODUCTION: Tendons are connective tissues essential for movement; however due to high loading demands and age-related degeneration, tendon pathologies are common and recurrence rate is high after treatment (1, 2). There is an unmet need for clinical therapies due to a lack of understanding of basic tendon biology. Despite the functional importance of tendon as a load-bearing tissue, the mechanotransduction pathways that govern tendon response to muscle loading throughout development and homeostasis remain almost completely unidentified. One well-established mechanotransduction pathway is MRTF/SRF. Primarily studied in the context of cardiac and skeletal muscle tissues, the MRTF pathway is modulated by microenvironmental mechanics that promote F-actin polymerization, enabling MRTF nuclear translocation and partnering with SRF to mediate transcription (3). In the context of tendon mechanobiology, the role of MRTF/SRF is unknown. To address this knowledge gap, we inhibited MRTF signaling during mouse embryonic stem cell (mESC) tenogenesis as a model of embryonic fibrous tissue development. We hypothesized that MRTF/SRF signaling is required for tendon induction and differentiation.

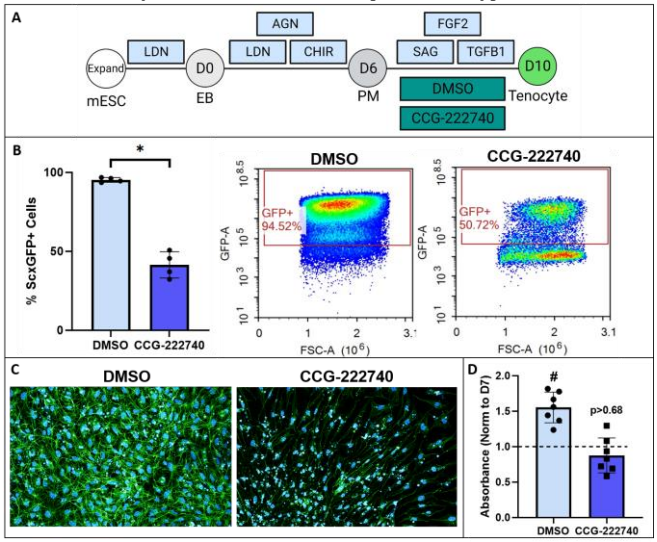

Fig 1. MRTF inhibition resulted in lower induction efficiency of ScxGFP+ cells, reduced actin stress fibers, and reduced proliferation. **A:** Flow cytometry of ScxGFP+ cells. *p<0.05, n=4. **B:** Reduced F-actin staining. (Green: Phalloidin, Blue: DAPI) **C:** CCK-8 showed reduced proliferation, but not viability. #p<0.05 vs day 7 starting values (dashed line), n=7.

METHODS: To derive ScxGFP+ tenocytes, mESCs isolated from ScxGFP+ blastocysts were cultured in serum-free media as embryoid bodies, specified to paraxial mesoderm (CHIR-99021 (5uM), LDN-193189 (500nM), AGN-193109 (100nM)), and differentiated into ScxGFP tenocytes (TGFβ1 (10ng/mL), FGF2 (100nM), and SAG (10ng/mL)) using our previously published protocol (**Fig 1A**) (4). To assess the role of MRTF/SRF signaling pathway in tendon differentiation, cells were cultured with established MRTF inhibitor (CCG-222740, 10uM) or DMSO vehicle-control (5). Tendon induction was quantified via flow cytometry and qPCR. Inhibitor cytotoxicity was assessed by CCK-8 assay (Dojindo). Statistical analyses were performed with ANOVA or student's T-tests with significance set at p<0.05.

RESULTS: At day 10 of differentiation, inhibition of MRTF/SRF with CCG-222740 resulted in consistent inhibition of SRF target genes such as *Acta2* (data not shown). Flow cytometry showed significantly reduced induction of ScxGFP+ tenocytes (**Fig 1B**). Phalloidin staining for F-actin also revealed dramatic reduction of stress fibers with MRTF inhibition (**Fig 1C**). CCK-8 analysis showed significantly fewer cells in the inhibitor treated group compared to DMSO control at day 10, but not compared to the initial starting day 7 values, suggesting that MRTF inhibition blocked proliferation but did not cause cytotoxicity (**Fig 1D**). While the tendon progenitor marker *Scx* was not affected at day 10, tendon differentiation markers (*Tnmd, Mkx, Col1, Lox)* were significantly decreased, consistent with impaired tenogenesis (**Fig 2, 3**). Since we previously showed that TGFβ1+SAG induces both tendon and fibrocartilage cells in the absence of retinoic acid modulators (4), we also tested cartilage marker expression and found no change in progenitor marker *Sox9* but reduction in cartilage differentiation markers (*Acan*, *Col2*), suggesting that fibrochondrogenesis is similarly impaired (**Fig 2, 3**).

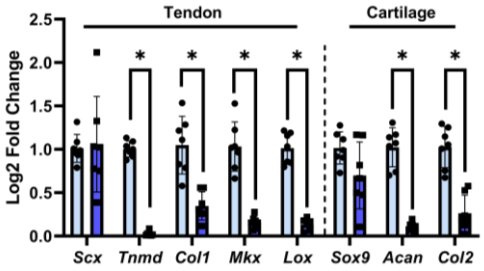

Fig 2: Reduction of differentiation markers with MRTF inhibition. Gene expression showed no difference in tendon/cartilage progenitor markers with decreased differentiation markers. *p<0.05, n=7.

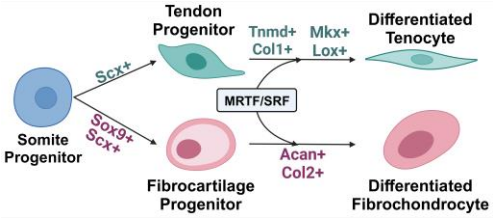

Fig 3: Proposed role for MRTF/SRF in tendon/fibrocartilage differentiation. MRTF/SRF signaling is important for tendon and fibrocartilage progenitor differentiation, but not initial specification.

DISCUSSION: These data establish a novel role for the MRTF/SRF signaling pathway as a regulator of tenocyte and fibrochondrocyte differentiation. Interestingly, inhibition of MRTF/SRF pathway did not significantly reduce expression of early tendon and cartilage markers associated with the induction of progenitors. However, inhibition significantly reduced markers associated with later differentiation stages for both tendon and fibrocartilage. In the mouse embryo, the appearance of loosely organized but patterned *Scx*+ tendon progenitors at E12.5 is followed by the onset of differentiation markers at E13.5. In the absence of muscle (Sp^d mice), E12.5 progenitors are normal but E13.5 differentiated cells are completely lost in the forearm and dramatically reduced in the paw (6). Our results suggest that the MRTF/SRF signaling pathway may regulate this critical response to early static muscle loading as a signal for tendon differentiation (7). While the role of muscle loading is less established for fibrochondrocytes such as those in the annulus fibrosus or meniscus, reports using the paralyzed muscle mutant muscular dysgenesis (*mdg*) showed impaired AF development and loss of meniscus structures in the absence of active muscle loading (8, 9). Based on our results, we propose the MRTF/SRF mechanotransduction pathway is an important mediator of force-mediated fibrous tissue differentiation during embryonic development. Ongoing studies will test the role of MRTF/SRF signaling *in vivo* through tissue-specific deletion of *Mrtfa* and *Mrtfb*.

SIGNIFICANCE: Identifying mechanotransduction pathways involved in tendon development will expand knowledge of basic tendon biology and can inform future tissue engineering and regenerative efforts.

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