Non-Muscle Myosin II Knockdown Disrupts Tenocyte Morphology and Contractility

Elizabeth R. Bernstein[1], Mary Kate Evans[1], Xi Jiang[1], Timur B. Kamalitdinov[1], Robert L. Mauck[1], Nathaniel A. Dyment[1]
[1]Dept. of Bioengineering, University of Pennsylvania, Philadelphia, PA
elizabeth.bernstein@pennmedicine.upenn.edu

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Introduction: Mechanical loading at physiologic levels is essential to the normal development, homeostasis, and repair of tendon[1]. It is well established that resident tenocytes are mechanoresponsive, but the mechanisms by which these cells sense, transmit, and respond to mechanical stimuli are still unclear[2]. The interaction between actin fibers and non-muscle myosin II (NM-II) is essential for force generation within cells[3,4]. Non-muscle myosin IIA and IIB, encoded by the genes *Myh9* and *Myh10*, are known to drive morphological changes in epithelial force-generating tissues[5,6]. Within tendon cells, we recently found that acto-myosin contractility mediates extracellular matrix remodeling through Yap/Taz/TEAD-mediated transcriptional activity[7]. The purpose of this study was to clarify the role of non-muscle myosin in tendon cell morphology and contractility using cells isolated from *Myh9*/*Myh10* double-floxed mice. The toxicity and variability of adenoviral vectors are a common pitfall of *in vitro* knockdown models. To address this challenge, we utilized a novel method of *in vitro* recombination, employing recombinant Cre protein modified to include a TAT cell-penetrating peptide in addition to a nuclear localization sequence.

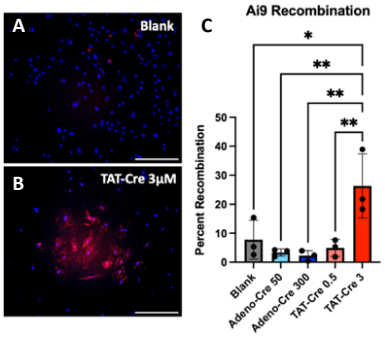

Fig 1. Representative images of (A) control cells and (B) TAT-Cre treated cells. Scale bars = 200μm. (C) Percent Ai9 recombination. * $p \leq 0.05$. ** $p \leq 0.01$.

Methods: All animal work was IACUC approved. Cell isolation: Tail tendons were dissected from Ai9 Rosa-tdTomato Cre reporter mice (n=3) or *Myh9$^{f/f}$;Myh10$^{f/f}$* mice (n=4) and digested in 2% collagenase IV/1.5% dispase II. Cell culture: Tendon cells were expanded and passaged in growth media. TAT-Cre treatment: Cells were treated with TAT-Cre at 0.5μM or 3μM for 5 hours in basal media without FBS. Adeno-Cre treatment: Cells were transduced with adeno-Cre (Ad5-CMV-Cre) at an MOI of 50 or 300 for 24 hours. To maximize infection efficiency, basal media was supplemented with 5μM polybrene. Recombination rate quantification: Twenty-four hours after treatment, tdTomato signal was assessed in live Ai9+ cells using inverted fluorescence microscopy. Immunofluorescence: *Myh9$^{f/f}$;Myh10$^{f/f}$* cells were seeded on fibronectin-coated glass coverslips. Two days after treatment, cells were fixed in 4% PFA, permeabilized with 0.1%Triton-X/PBS, stained with anti-paxillin (1:200) and phalloidin (1:100) then counterstained with DAPI. Morphology analysis: Cell area and solidity were quantified using CellProfiler. Explant model: Individual tendon fascicles were isolated from *Myh9$^{f/f}$;Myh10$^{f/f}$* mice (n=4) and cut to a length of approximately 15mm. Free floating explants were cultured in growth media supplemented with 50μg/mL ascorbic acid in 12-well plates. Explants were treated with TAT-Cre, blebbistatin, or nothing. The TAT-Cre group was incubated with 3μM TAT-Cre for 5 hours on days 1, 4, and 7. The blebbistatin group received fresh media with 10μM blebbistatin every 3 days for the duration of the experiment. Live/Dead assay: After 16 days, explants were incubated with 2μM calcein AM and 4μM EthD-1 for 30 minutes and imaged using inverted fluorescence microscopy. Statistics: Treatment groups for monolayer experiments and the live/dead assay were compared using a one-way ANOVA with Tukey post-hoc tests (α=0.05). Explant groups were compared using a repeated measures two-way ANOVA with Tukey post-hoc tests (α=0.05).

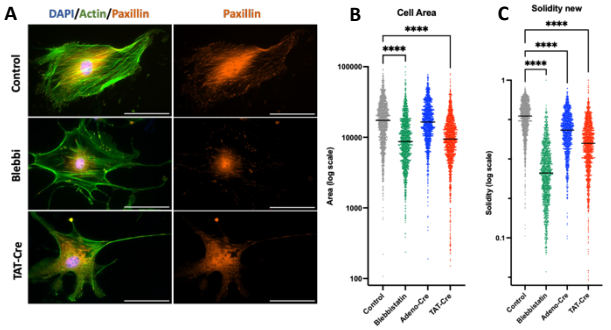

Fig 2. (A) Representative images of *Myh9$^{f/f}$;Myh10$^{f/f}$* cells. Scale bars = 100μm. (B) Cell area and (C) solidity. **** $p \leq 0.0001$.

Results: TAT-Cre induces recombination of tendon cells in monolayer. Incubation with 3μM TAT-Cre resulted in significantly higher Ai9 recombination than control cells (p=0.02) (**Fig. 1A-C**). The average recombination rate of cells treated with 3μM TAT-Cre was 26.32% (SD=11.03%) compared to 7.79% (SD=6.72%) among control cells. Neither concentration of Adeno-Cre resulted in effective recombination, even with previous concentrations used successfully by our group (**Fig. 1C**). NM-II knockdown disrupts stress fiber and focal adhesion formation. Based on the recombination rates seen in Ai9 cells, only the higher doses of Adeno-Cre and TAT-Cre were used for NM-II knockdown. *Myh9$^{f/f}$;Myh10$^{f/f}$* cells treated with TAT-Cre had disrupted morphology compared to control cells (**Fig. 2A**). NM-II knockdown resulted in decreased cell area ($p<0.0001$) and cell solidity ($p<0.0001$) (**Fig. 2B,C**). Additionally, TAT-Cre treatment disrupted focal adhesions (**Fig. 2A**). NM-II knockdown impairs tendon contractility. Given the lack of response to Adeno-Cre, functional outcomes were tested in an explant model using only TAT-Cre and the NM-II inhibitor, blebbistatin. Similar to blebbistatin, treatment with TAT-Cre disrupted the ability of tendon cells to contract the free-floating fascicle compared to control tendons (p=0.05). (**Fig. 3A,B**). Live/dead staining showed increased cell death in TAT-Cre-treated explants relative to control and blebbistatin groups, but these differences did not reach significance ($p>0.05$) (**Fig. 3C,D**).

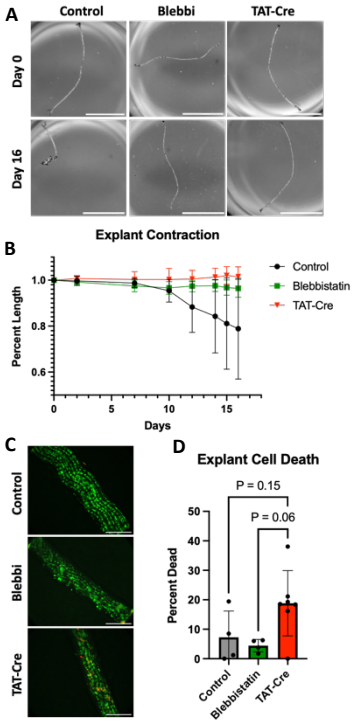

Fig 3. (A) Representative images of explants. Scale bars = 5mm. (B) Explant percent of initial length. (C) Representative images of live/dead staining of explants. Scale bars = 200μm. (D) % dead cells at day 16.

Discussion: This study demonstrates that TAT-Cre is an effective tool for inducing *in vitro* and *in situ* recombination of tendon cells. A concentration of 3μM was sufficient to induce Ai9 recombination in over 25% of cells without noticeable effects on cell morphology or viability. After confirming its efficacy, we used TAT-Cre to illustrate the vital role of NM-II in directing tendon cell morphology through stress fiber and focal adhesion formation. Within 48 hours of knockdown, cells showed altered morphology with decreased cell spreading and solidity. Furthermore, these data establish the necessity of *Myh9* and *Myh10* in tendon contractility as TAT-Cre-mediated gene excision in our explant model was equal to blebbistatin-mediated NM-II inhibition, although we are investigating the impact of the increased cell death in this finding. Future work will further tune treatment dose and duration, in order to minimize cell death, as well as confirm NM-II knockdown via qPCR and western blotting.

Significance/Clinical Relevance: TAT-Cre offers an innovative tool to investigate fundamental biological mechanisms within cells using Cre-Lox without time consuming and costly mouse breading with Cre lines.

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References: 1. Galloway, *J Bone Joint Surg*, 2013; 2. Lavagnino, *JOR*, 2015; 3. Murrell, *Nat Rev Mol Cell Biol*, 2015; Vicente-Manzanares, *Nat Rev Mol Cell Biol*, 2009; 5. Pecci, *Gene*, 2018; 6. Franke, *Curr Biol*, 2005; 7. Jones, *PNAS*, 2023.