

# Blocking Cav1.2 function by verapamil mitigates tendinopathy and promotes scarless healing of injured Achilles tendon in mice

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**Introduction:** Tensile-bearing Achilles tendons are highly prone to acute injury and chronic degeneration known as tendinopathy, a disease often associated with pain and disability. However, there is great unmet clinical need for novel therapeutic options as aberrant tendon healing such as scar formation and heterotopic ossification compromises tendon structure and function, whereas current standard of care for Achilles tendinopathy does not result in effective long-term functional recovery. The pathogenic mechanisms of Achilles tendinopathy are largely unknown, which prevents the development of new therapeutic strategies for tendinopathy. We recently demonstrated that Cav1.2, an L-type voltage-gated Ca<sup>2+</sup> channel is dynamically expressed in tendon and regulates tendon formation during development and postnatal growth<sup>1</sup>. Interestingly, our clinical study using TriNetX Analytics Network reveals bidirectional association between hypertension and Achilles tendinopathy, and the decreased incidence of Achilles tendinopathy in hypertensive patients who were on calcium channel blockers (CCBs) compared with those on other hypertension medication (data unpublished yet). Given the fact that dysregulation of Cav1.2 expression and activity contribute to hypertension<sup>2</sup>, we hypothesized that aberrant Cav1.2 function is a shared pathological mechanism which also underlies Achilles tendinopathy; blocking Cav1.2 function by CCBs mitigates this disease development. To test this hypothesis, we examined Cav1.2 expression in Achilles tendon in response to injury and tested the effect of upregulated Ca<sup>2+</sup> signaling using Cav1.2 transgenic mouse models on Achilles tendinopathy development and evaluated the efficacy of L-type specific CCB verapamil on Achilles tendinopathy in an injury-induced mouse model.

**Methods:** All animal studies were approved by the University Committee for Animal Resources. **Mice:** This study used two Cav1.2 transgenic lines (which either carry a wildtype or a G406R gain-of-function Cav1.2 mutant cDNA knocked into the Rosa26 locus with an upstream floxed stop codon; expression of the conditional Cav1.2<sup>WT</sup> or Cav1.2<sup>G406R</sup> allele was achieved by crossing with the *ScxCre* mouse), Cav1.2<sup>+/lacZ</sup> (which carries a lacZ reporter with a nuclear localization signal under the promoter of Cav1.2 gene) and C57BL/6J mice. Both male and female mice were used for analysis. **Partial unilateral Achilles tendon transection (PUAT):** 10-week-old Cav1.2<sup>+/lacZ</sup> or C57BL/6J mice were subjected to PUAT in the left leg. To investigate the effect of injury on Cav1.2 expression, tissues were collected from Cav1.2<sup>+/lacZ</sup> mice 7 days (D7) post-surgery, with the right uninjured Achilles tendon serving as the contralateral control. To evaluate the efficacy of CCB verapamil on mitigating the development of Achilles tendinopathy, PUAT C57BL/6J mice were divided into two groups: vehicle (DMSO diluted 1:3 in 1xPBS) vs verapamil (15 mg/kg/day, i.p.) treatments starting on the day of surgery for 42 days. Tissues were collected for analysis at D42 post-surgery. **X-gal staining:** Visualization of lacZ expression was done by whole-mount x-gal staining, followed by frozen sectioning (10 µm), counter-stained with nuclear fast red. **µCT analysis:** To monitor heterotopic bone formation upon injury, µCT 3D images were acquired using a Scanco VivaCT 40 instrument, either in vivo longitudinally or ex vivo following tissue harvest and 10% neutral buffered formalin fixation. **Histology:** Decalcified paraffin sections (5 µm) were stained with Alcian Blue, Hematoxylin/Orange G (ABOG).

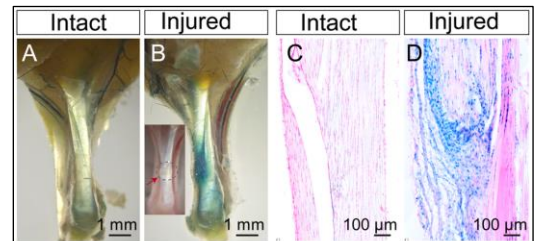
**Results:** **Acute injury induces Cav1.2 re-expression in Achilles tendon.** We found that PUAT of 10-week-old Cav1.2<sup>+/lacZ</sup> reporter mice induces substantial Cav1.2 re-expression, supported by a significant increase of x-gal-stained tendon cells around the injury site at D7 post-injury, in striking contrast with the uninjured adult Achilles tendon, which has very limited Cav1.2 expression in tenocytes (Fig. 1). **Tendinopathy (including ectopic bone) is developed in Achilles tendons of Scx-Cav1.2<sup>WT</sup> and Scx-Cav1.2<sup>G406R</sup> mice.** Longitudinal µCT analysis showed that Scx-Cav1.2<sup>WT</sup> mice develop ectopic bone in Achilles tendons by 3-4 months of age in both male and female mice with 100% penetrance in the absence of injury. This phenotype is significantly accelerated in Scx-Cav1.2<sup>G406R</sup> mutant mice with early onset (~2 months old) and increased ectopic bone formation. Ectopic bone progresses in Achilles tendons with age in both transgenic mouse models. ABOG staining shows that tendinopathy in Achilles tendon is accompanied by chondrocyte (trans)differentiation in the middle tendon substance as well as in the lesion of ectopic bone. **PUAT induces Achilles tendinopathy and ectopic bone formation.** We found PUAT of 10-week-old C57BL/6J mice induces Achilles tendinopathy with ectopic bone formation visualized by µCT analysis. Histological ABOG reveals scar tissue formation in the gap of the injury site while neighboring intact tendons undergo intense chondrocyte transdifferentiation and extracellular matrix (ECM) degeneration (Fig. 2A-C). **CCB verapamil mitigates tendinopathy and promotes scarless healing of PUAT-injured Achilles tendon in C57BL/6J mice.** Achilles tendons of verapamil-treated C57BL/6J mice D42 post PUAT show no obvious gap in the injured site, compared with those of vehicle-treated mice (Fig. 2A & 2D). µCT analysis reveals reduced ectopic bone formation in the injured Achilles tendons when mice are subjected to verapamil treatment (1 out of 4 mice forms ectopic bone vs 4 out of 4 in vehicle treated group) (Fig. 2B & 2E). ABOG staining further revealed that verapamil promotes scarless healing with a better organized ECM matrix in the injury site (more glycosaminoglycan composition in ECM instead of scar matrix) and intact neighboring tissue without obvious ECM degradation, although chondrocyte transdifferentiation is also initiated.

**Discussion:** In this study, using Cav1.2<sup>+/lacZ</sup> reporter mice we found that Cav1.2 expression is upregulated early in response to inflammation upon PUAT. We also observed that PUAT induces Achilles tendinopathy with scar formation, neighboring tendon tissue degeneration, and heterotopic bone formation in wild-type mice. These data provide a rational linkage among high Cav1.2 expression, injury-induced inflammation, and Achilles tendinopathy. Consistently, our transgenic mouse models demonstrated that maintaining high expression of Cav1.2<sup>WT</sup> or Cav1.2<sup>G406R</sup> mutant channel in tendon is sufficient to induce Achilles tendinopathy, providing a proof-of-concept that aberrant Cav1.2 expression and activity is implicated in tendinopathy development. Furthermore, pilot data showed that blocking Cav1.2 activity with verapamil mitigates injury-induced Achilles tendinopathy progression and promotes scarless tendon healing, in line with data from our clinical study demonstrating CCBs taken by hypertensive patients reduce the incidence of Achilles tendinopathy.

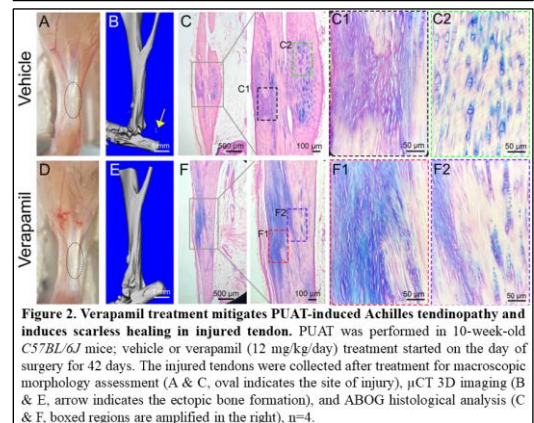
**Clinical Significance:** Our study identifies a novel role of Cav1.2 channel and its mediated Ca<sup>2+</sup> signaling which underlies the pathogenesis of Achilles tendinopathy and the efficacy of CCB verapamil to alleviate the progression of Achilles tendinopathy. These data provide a scientific rationale for repurposing the use of FDA-approved generic CCBs to prevent or treat Achilles tendinopathy.

## References:

1. Li H, Korcari A, Ciufo D, et al. Increased Ca(2+) signaling through Ca(V)1.2 induces tendon hypertrophy with increased collagen fibrillogenesis and biomechanical properties. *Faseb j*. 2023;37(7):e23007.
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**Figure 1.** Acute injury induces Cav1.2 re-expression in adult Achilles tendon. PUAT was performed in 10-week-old Cav1.2<sup>+/lacZ</sup> mice with analysis carried out at D7 post injury. (A and B) Whole-mount x-gal staining for contralateral intact and PUAT injured Achilles tendons. Inset shows the macroscopic morphology of injured tendon at D7. (C and D) Frozen sections (10 µm) of x-gal stained contralateral intact and PUAT injured Achilles tendons counterstained with nuclear fast red. n=3. Note: lacZ gene carries a nuclear location signal. Thus, x-gal staining (blue color) was restricted in the nucleus.



**Figure 2.** Verapamil treatment mitigates PUAT-induced Achilles tendinopathy and induces scarless healing in injured tendon. PUAT was performed in 10-week-old C57BL/6J mice; vehicle or verapamil (12 mg/kg/day) treatment started on the day of surgery for 42 days. The injured tendons were collected after treatment for macroscopic morphology assessment (A & C, oval indicates the site of injury), µCT 3D imaging (B & E, arrow indicates the ectopic bone formation), and ABOG histological analysis (C & F, boxed regions are amplified in the right). n=4.