Characterization of natural aging in mouse Achilles tendons

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INTRODUCTION: Age-related changes in tendon include alterations to the extracellular matrix (ECM) structure and functional deficits[1]. As tendons are critical for movement, these degenerative changes limit successful mobility and therefore reduce quality of life in aged individuals. Previous mouse studies in the flexor digitorum longus (FDL) tendon have demonstrated decreased total cell density but no mechanical deficits during natural aging[2]. Whether or not these age-related changes are conserved in other tendons is unknown. Therefore, the goal of the present study was to comprehensively characterize the effects of natural aging in mouse Achilles tendons. Given that anatomically different young mouse tendons have distinct functions and are transcriptionally different[3], we hypothesized that aged mouse Achilles tendons will have distinct age-related changes compared to aged mouse FDL tendons.

METHODS: The University Committee on Animal Resources approved all studies. *Mice*: Given that age-related changes in tendon are more prevalent over the age of 40 in humans[1], we evaluated Achilles tendons of 18-month-old male (C57BL/6J) mice (56-69 years in humans[4]) (National Institute of Aging [NIA]). 10- month male (C57BL/6J) mice (NIA) were used to represent a middle-aged timepoint (38-47 years in humans[4]). 4-month old male (C57BL/6J) mice (20-30 years in humans[4]) were used as young controls (Jackson Laboratory). *Biomechanical assessment*: Achilles tendons were carefully dissected and gripped between two clamps. Cross-sectional area (CSA) was calculated from three measurements of tendon width and thickness using ImageJ. Achilles tendon then underwent tensile testing using a customized uniaxial micro tester (eXpert 4000 Microtester and MTESTQuattro software). Tendons were then preloaded to 0.1N to remove slack, followed by 10 cycles of pre-load at 6% strain, and finally ramp-to-failure testing at 1 mm/s (n=10-15 per group). Force-displacement and stress-strain were plotted and analyzed to determine mechanical (stiffness and peak load at failure) and material (elastic modulus and peak stress) properties. *Histology*: Hindlimbs were fixed in 10% formalin for 72 hours and decalcified with Webb-Jee EDTA for 14 days (n=3-4 per group). Three-micron sagittal sections were cut and stained with Alcian Blue Hematoxylin and Orange G (ABHOG) or with Hoechst 33342. *Quantification of total cell density*: Hoechst 33342 stained nuclei were quantified using VIS Image Analysis Software (Visiopharm) and normalized to total area. *Micro-CT (µCT) Imaging and Quantification of bone formation*: Hindlimbs were dissected and fixed in 10% formalin for 72 hours (n=3-4 per group). Following fixation, hindlimbs were imaged with µCT (VivaCT 40, Scanco Inc.) The total tissue volume of the entire soft tissue region above the calcaneus, including bone deposits of these regions was measured to calculate the bone volume fraction (BV/TV) (Scanco Software). *Statistics*: Differences in biomechanics, total cell density and bone volume fraction were assessed by unpaired t-tests and one way ANOVA (GraphPad Prism).

RESULTS: *18-month old Achilles tendons have increased mechanical properties compared to 4-month old controls*: No differences in CSA or peak load were detected between 4- and 18- month old Achilles tendons (Fig. 1A, B). 18-month Achilles tendons had a ~15% increase in stiffness (p=0.02), a ~29% increase in peak stress (p=0.0014) and a ~60% increase in elastic modulus (p<0.0001), compared to young controls (Fig. 1C-E). These data suggest that 18-month old Achilles tendons have altered function compared to young controls. *No changes in total tendon cell density between 4-, 10- and 18- month old Achilles tendons*: No significant differences in total cell density were detected between 4-, 10- and 18- month old Achilles tendons (Fig. 2A). These data suggest that cells are not lost during natural aging. *10- and 18- month old Achilles tendons exhibited altered tendon cell morphology and ECM composition:* We observed rounder, enlarged tendon cells with more proteoglycan (blue) staining in the 10- and 18- month Achilles tendons, compared to 4-month Achilles tendons (Fig. 2B). Interestingly, 50% of 18-month old Achilles tendon samples exhibited a large protrusion of tissue on the anterior side of the tendon, which is not present in 4- and 10- month old Achilles tendons (Fig. 2B). This suggests that 18-month old tendons exhibit morphological changes in tendon cells and altered ECM composition. *10- and 18- month old Achilles tendons have substantial bone volume fraction compared to 4- month old controls*. Upon dissection of 18-month old Achilles tendons during mechanical testing, we noticed small areas of apparent ossification in the tendon. Therefore, we imaged 10- and 18- month old Achilles tendons with µCT and compared to 4- month controls (Fig. 3A). 10- and 18- month old Achilles tendons demonstrated significant bone volume fraction, compared to 4-month old controls (10-month BV/TV: 0.04213 ± 0.01475, p=0.0079; 18-month BV/TV: 0.4540 ± 0.01808, p=0.0053) (Fig. 3B). These data indicate that heterotopic ossification (HO) was present as early as 10-months old in mouse Achilles tendons.

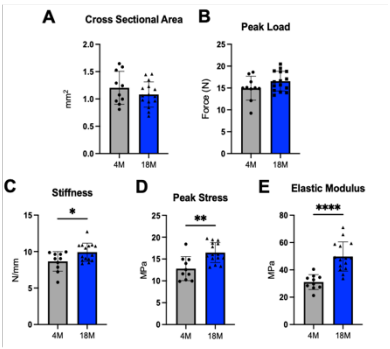

Fig. 1: Biomechanical assessment revealed no significant differences in (A) CSA or (B) peak load between VWR and underloaded controls. Significant increases were detected in (C) stiffness, (D) peak stress and (E) elastic modulus in VWR tendons relative to underloaded controls (n=10-15 per group). *p≤0.05, **p≤0.01, ****p≤0.0001

DISCUSSION: It is well known that during aging tendon structure and function can become impaired. The results of our study show significant biomechanical alterations but no changes in total cell density, which is in direct contrast to previous FDL tendon studies[1]. Our µCT data shows that 10- and 18-month old Achilles tendons exhibit significant bone formation, compared to 4-month old controls. To our knowledge, bone formation in aged FDL tendons has not been reported, which suggests that age-related HO may be specific to Achilles tendons. Given that there are no changes in total density, the abnormal tendon cell morphology and ECM, resident tendon cells may play a role in Achilles tendon HO. Taken together, our data suggests that mouse Achilles tendons have distinct age-related hallmarks, compared to mouse FDL tendons. It is critical to understand if anatomically distinct tendons have different age-related changes, as this would suggest the need for specific therapies for different tendons during aging. Future studies are assessing the cellular processes that are responsible for these divergent aging phenotypes, as well as the age-related changes in female Achilles tendons.

SIGNIFICANCE: Understanding different aging processes between anatomically distinct tendons can help identify tendon-specific therapeutic targets mitigate age-related changes.

REFERENCES: 1. Korcari et al. 2023 2. Ackerman et al. 2017 3. Disser et al. 2020 4. Flurkey et al. 2007

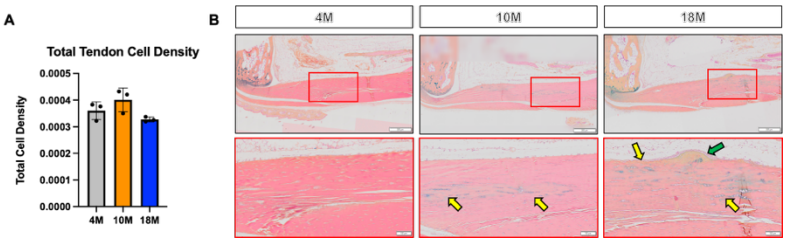

Fig 2: (A) No significant differences in total tendon cell density were observed between 4-, 10- and 18-month old Achilles tendons. (B) Qualitative analysis of Alcian Blue Hematoxylin and Orange G (ABHOG) staining of 10- and 18- month Achilles tendons demonstrate more proteoglycan expression (blue staining) and enlarged tendon cells (yellow arrows), compared to 4-month old controls. An abnormal protrusion of tendon tissue is present in 18-month old tendons (green arrow), which is not present at 4- or 10- months.

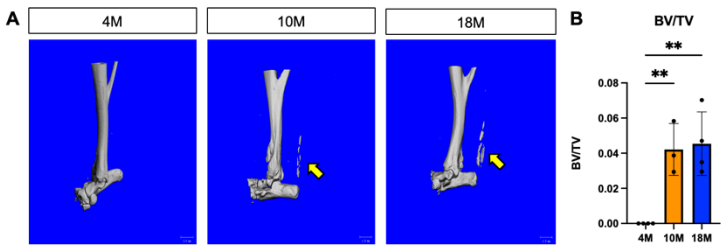

Fig. 3: (A) µCT images of 4-, 10-and 18-month-old Achilles tendons (yellow arrows). (B) Quantification of bone volume fraction (bone volume/total volume) [BV/TV] between 4-, 10- and 18-month-old tendons (n=3-4). **p≤0.01.