A new mechanistic insight into the pathogenesis of Achilles tendinopathy and heterotopic ossification by study of human patients

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Introduction: Achilles tendinopathy is a common cause and disability among recreational athletics, physical workers and the elderly, with high socio-economic impact[1]. In our TriNetX analysis, about 0.5% of the general population developed Achilles tendinopathy. A frequent late manifestation of this disease is tendon heterotopic ossification (HO), most arising via endochondral ossification, wherein chondrocyte differentiation precedes replacement by osteoblasts. Despite widespread use of immobilization, NSAIDS, corticosteroid injections and physical therapy[2], there are no definitive disease-modifying treatments for Achilles tendinopathy and HO; surgery to remove pathological tissue is often required, yet recurrence or progression can occur. The cellular and molecular mechanisms underlying this pathogenetic condition remain elusive, limiting the development of new therapeutic strategies. While animal models provide mechanistic insight, surgically discarded human Achilles tendon offers the most direct and clinically relevant substrate, capturing the chronicity and mineralization patterns of real disease. Here, we investigate surgically excised human Achilles tendons to define the cellular and molecular programs underlying tendinopathy and tendon HO, with the goal of identifying human-relevant targets for intervention.

Methods: The study using surgically excised human Achilles tissue was approved by the University's Institutional Review Board. ***Specimens***: Surgically excised Achilles tendon from 9 patients (50~73 years old, both sexes); control Achilles tendons from 3 healthy donors (Articular Engineering). ***Alcian Blue Hematoxylin/Orange G (ABOG) and tartrate-resistant acid phosphatase (TRAP) analyses:*** Tissues were fixed in 10% neutral buffered formalin, decalcified, paraffin processed, and sectioned at 7 µm. ABOG staining was performed on paraffin sections; TRAP staining was carried out on serial sections using standard protocol. ***Immunofluorescence (IF) analysis*:** Paraffin sections were incubated with primary antibodies against RUNX2 (Sigma, ZRB1603), ALPL (Proteintech, 83767-1-RR), and TNC (Proteintech, 11187-1-AP), followed by Alexa fluor 647-conjugated secondary antibodies to detect signal and minimize autofluorescence; nuclei were counterstained with DAPI. ***Imaging and Mapping:*** Whole-slide images were acquired on an Olympus VS120 Virtual slide scanner. After IF imaging, the same slides were counterstained with ABOG to map IF-positive cells to corresponding histologic compartments.

Results:

Histological findings: We examined 9 surgical cases from University of Rochester Medical Center clinic that display obvious radiographic Achilles tendon calcification underwent surgery. ABOG staining demonstrated mature lamellar bone in all specimens, with well-formed osteons and marrow cavities showing vascularization but little residual hematopoietic marrow (**Fig.1**). The ectopic bone was contiguous with adjacent tendon displaying matrix alterations, for example, increased cartilage glycosaminoglycan content and the appearance of chondrocyte-like cells. These cells often align along the native tendon fiber axis, consistent with derivation from tenocytes. Tendon remnants (Alcian blue-positive) were frequently trapped within the mature bone in all cases (**Fig.1**) although the size of the remnants varied among different loci and individuals. Furthermore, TRAP staining identified active osteoclasts beneath tendon remnant and the ectopic bone tissue (**Fig. 2**). Weak TRAP staining was also detected in some chondrocyte-like cells of the trapped tendon tissue. These data indicate ongoing bone remodeling even at late stage of Achilles HO.

Chondro-Osteogenic commitment: Immunofluorescence revealed nuclear RUNX2 protein in a subset of linearly aligned, Alcian blue-positive chondrocyte-like tenocytes, in contrast to the spindle-shaped tenocytes of healthy Achilles tendon (**Fig. 3**). Furthermore, ALPL protein was also detected in the altered tendon regions with increased cartilage -matrix expression and in some trapped tendon remnants where ALP localized around Alcian blue-positive chondrocyte-like cells. Consistently, TNC expression was minimal within tendon remnants and the adjacent altered tendon tissue.

Discussion:

In surgically excised human Achilles tendons with radiographic calcification, we detected a continuous spectrum from altered tendon to mature lamellar bone. The altered tendon regions showed Alcian blue–positive cartilage-like matrix and linear arrays of chondrocyte-like cells oriented along the native tendon fiber axis, accompanied by increased RUNX2 and ALPL and reduced TNC protein. TRAP-positive osteoclasts at the tendon-bone interface, together with TRAP-positive chondrocyte-like cells in tendon remnants embedded within mature bone, indicate active remodeling even at late stages. Collectively, these features are most consistent with an endochondral, rather than intramembranous, ossification of ectopic bone formation in Achilles tendon. We propose a working model in which tenocytes undergo chondrogenic transdifferentiation, depositing a cartilage-like matrix that subsequently mineralizes; this calcified cartilage is then remodeled by osteoclasts and/or chondrocyte-like cells, and replaced by lamellar bone, sometimes trapping residual tendon within the ossified mass. We refer to this sequence as teno-chondral ossification, emphasizing its origin in tendon and its chondrogenic intermediate. This study has limitations. The specimens represent end-stage, surgically treated disease and provide cross-sectional (not temporal) snapshots; thus, marker expression (RUNX2, ALPL) supports commitment but does not establish lineage or causality. The cohort is modest, and alternative progenitor sources (e.g., tendon-resident progenitors) cannot be excluded. Definitive proof of tenocyte-to-chondrocyte fate conversion will require lineage tracing in animal models, with temporally controlled tenocyte reporters, and complemented by time-course analyses to map intermediate states and matrix transitions.

Clinical Significance: Despite limitations in this study, our findings outline testable checkpoints, including tenocyte chondrogenic transition, cartilage-matrix deposition, mineralization, and osteoclastic remodeling. These may serve as therapeutic entry points. Interventions that disrupt the chondrogenic phase or modulate remodeling could plausibly attenuate disease progression. In summary, Achilles ectopic bone formation appears to follow an endochondral trajectory originating within tendon, for which we propose the term teno-chondral ossification.

Reference:

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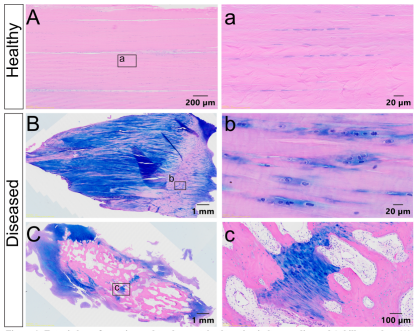

Figure 1: Ectopic bone formation and tendon matrix alteration in human diseased Achilles tendon. (A) ABOG (Alcian blue/Orange G) staining of healthy Achilles tendon shows normal collagenous matrix (pink) with spindle-shaped tenocytes; boxed area shown at higher magnification in (a). **(B&C)** Diseased Achilles tendon displays Alcian blue-positive, glycosaminoglycan-rich matrix and linear arrays of chondrocyte-like cells aligned with the native tendon fiber axis adjacent to ectopic bone; mature lamellar bone with osteonal architecture is continuous with theses altered regions, and Alcian blue-positive tendon remnants are entrapped within the bone. Boxed areas are shown at higher magnification in (b) and (c), respectively. n = 3 for healthy Achilles tendon, and n = 9 for diseased Achilles tendon.

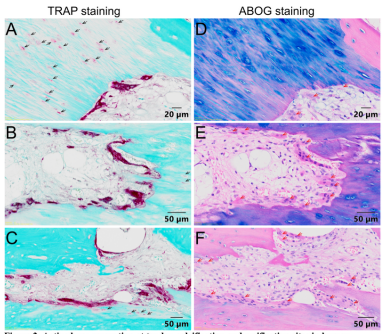

Figure 2: Active bone resorption at tendon calcification and ossification sites in human diseased Achilles tendon. (A-C) TRAP staining highlights multinucleated, TRAP-positive osteoclasts located beneath Alcian blue-positive tendon remnants entrapped within the lesion (A, B) and the surface of ectopic lamellar bone (C). Weak TRAP activity is also observed in chondrocyte-like cells (black arrows). **(D-F)** Serial sections stained with ABOG from the corresponding fields in (A-C) delineate tissue compartments, mapping osteoclasts to calcified tendon remnants and mineralized bone surfaces (red arrows), and situating chondrocyte-like cells within glycosaminoglycan-rich matrix.

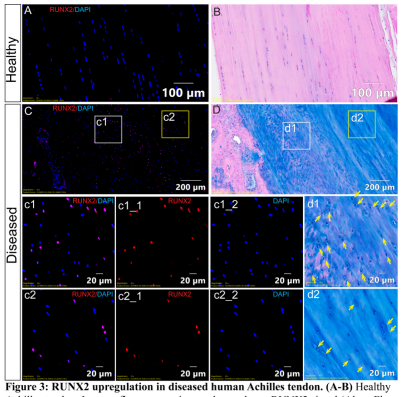

Figure 3: RUNX2 upregulation in diseased human Achilles tendon. (A-B) Healthy Achilles tendon: Immunofluorescence image shows absent RUNX2 signal (Alexa Fluor 647) (A); the corresponding field stained with ABOG (B) displays normal collagen matrix. **(C-D)** Diseased Achilles tendon: robust nuclear RUNX2 signal (C) in cells that, on the corresponding ABOG-counterstained same section (D), localized within Alcian blue-positive, chondrocyte-like regions and mature bone area. Boxed regions are shown at higher magnification in c1, c2, d1, and d2. Singla-channel separations (RUNX2 /Alexa-647 and DAPI) are presented in c1_1, c1_2, c2_1, and c2_2. Yellow arrows mark RUNX2-positive chondrocyte-like cells in the ABOG-stained section.