Primary Cilia Are Highly Oriented with Respect to Collagen Direction and Long Axis of Extensor Tendon

INTRODUCTION
Primary cilia are microtubule-based sensory organelles that project from the cell surface into the extracellular environment. They are known to participate in mechanotransduction processes in bone cells and have been hypothesized to play a role in oriented secretion of extracellular matrix (ECM) molecules in skeletal tissues [1, 2]. However, the mechanisms by which cells sense forces and respond with signals for tissue formation and remodeling are incompletely understood. Before hypotheses about the role of the primary cilium in tissue modeling can be explored, ciliary incidence and orientation need to be analyzed in native skeletal tissues. In this study, primary cilia were investigated in tendon, a tissue with a highly oriented collagenous matrix ideal for analysis of spatial relationships between primary cilia and the ECM. The objective of this study was to characterize the incidence and orientation of tenocyte primary cilia in their native ECM. We hypothesized that tenocytes would have one primary cilium per cell, as has been shown previously for articular chondrocytes [3], and that primary cilia would be aligned with the collagen fiber direction in the tendon.

METHODS
Primary cilia, tenocyte nuclei, and collagen were characterized in immunofluorescently labeled whole fasicles and sections using multiphoton microscopy (MPM). Digital extensor tendons were collected from 3-week-old Sprague-Dawley rats under an IACUC-approved protocol. For the analyses of ciliary orientation, whole fasicles from 10 rats were fixed in methanol, immunolabeled with acetylated α-tubulin primary antibody, and stained with Hoechst. For the analyses of ciliary incidence, formalin-fixed tendons from 6 rats were embedded in paraffin, and 5-μm-thick sections were immunolabeled as described above. The MPM system, described previously [4], comprised a Ti:Sapphire laser generating 100-fs pulses at 800 nm and a 40x/1.15 NA objective. Three signals were collected: endogenous second harmonic generation from the collagen, green fluorescence from the primary cilia, and blue fluorescence from the nuclei. Z-stacks were collected as the laser was focused into the tissue in 1-μm steps, deconvolved, and reconstructed for 3D visualization (Fig. 2b). The image stacks from the sections were analyzed to determine the ratio of nuclei with cilia to total nuclei. For each animal, one full-thickness stack was collected from each of two sections; nuclei on the edges of the stack were excluded from the analysis. The image stacks from the whole fasicles were analyzed with a quantitative image-processing algorithm in which nuclei and cilia were modeled as ellipsoids and curves, respectively [5]. Outcome parameters included the ciliary length, the in-plane angle θ with respect to the proximal-distal (y) axis, and the elevation angle φ with respect to the cranial-caudal (z) axis (Fig. 2a).

RESULTS
Primary cilia, tenocyte nuclei, and collagen were visualized in situ throughout sections and whole fasicles. Tenocyte nuclei lay between the collagen fiber bundles, with the long axes of the nuclei parallel to the collagen direction (Fig. 1a,b). The ratio of nuclei associated with a primary cilium to total nuclei was 0.64 ± 0.07 (mean ± SD, n=304).

Cilia were highly oriented with respect to the collagen and the long axis of the tendon. The distribution of the ciliary elevation angle had a peak near 90°, indicating that the cilia lay primarily in the x-y (frontal) plane (Fig. 2c). The mean elevation angle φ was 74.1±13.0° (n=49). The distribution of the in-plane angle θ had peaks near 90° and 270°, indicating that the in-plane components of the cilia lay parallel to the collagen direction (Fig. 2d). The mean ciliary length was 4.4 ± 2.4 μm.

DISCUSSION
The goal of this study was to characterize the incidence and orientation of primary cilia in native tendon ECM. Efficient optical sectioning and 3D analysis of primary cilia, tenocyte nuclei, and collagen were achieved throughout tendon tissues with MPM. The use of sections for the incidence analysis ensured maximal antibody penetration, while the use of whole fasicles for the orientation analysis allowed optimal preservation of the 3D tissue structure. Ciliary incidence was 64%, similar to corresponding values of 61-62% observed in cultured MC3T3-E1 osteoblasts and MLO-Y4 osteocytes [1]. Although all cells are expected to have one primary cilium [3], the presence of primary cilia is cell-cycle dependent, with cilia present only during interphase [6].

Primary cilia were preferentially oriented with respect to the ECM. Ciliary axonemes predominantly lay in the x-y (frontal) plane, oriented parallel to the collagen direction and the long axis of the tendon. Interestingly, this axis is also the primary tensile loading direction of the tendon. Because primary cilia may play a role in establishing cell orientation and secreting ECM components in response to mechanical stimuli [2], the observed orientation distribution of the cilia relative to the collagen may reflect an adaptation to the loading environment. The data in the current study will serve as baseline values reflecting the normal loading environment, and our approach can now be extended to analyze the 3D orientation of primary cilia under altered loading regimes. Our methodologies will thus allow further exploration of the role of the primary cilium in mechanotransduction and establishment of ECM organization in skeletal tissues.

REFERENCES

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