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Primary cilia as the nexus of biophysical and hedgehog signaling at the tendon enthesis

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INTRODUCTION

Tendon and its associated enthesis are essential components of the musculoskeletal system, linking muscle to bone to allow for coordinated muscle contraction, skeletal movement, and stability (1). This critical connection between muscle and bone, however, is prone to injury, accounting for a large number of musculoskeletal injuries in the United States every year (1–3). These injuries are often the result of overuse damage or undergo degeneration, leading to tendinopathy, enthesopathy, and eventually tissue rupture. Despite the prevalence of these injuries, no consensus treatments are available to prevent tendinopathy or enthesopathy or to recover a torn tissue to its native structure and full biomechanical functionality. There remains a critical gap in knowledge in our understanding of tendon enthesis responses to loading and the biological mechanisms that control these responses. The development of effective therapeutics is therefore hampered by our limited knowledge of tendon enthesis development biology, mechanobiology, and the endogenous mechanisms governing tendon pathogenesis and healing (3).

Both mechanical force and hedgehog (Hh) signaling are necessary for the development and maintenance of the tendon enthesis (4,5). Loading deprivation of mouse shoulders during postnatal development causes structural, compositional, and functional defects in the tendon enthesis, including reduced collagen fiber alignment, decreased mineral content, and impaired mechanical function (6). Conversely, application of moderate in vivo loading by treadmill running in both adult and aging mice shows beneficial effects, including increased gene expression related to tenogenesis, enhanced mechanical properties, and altered tendon composition (7). Hh signaling also drives enthesis fibrocartilage formation, mineralization, and healing. A unique population of Hh-responsive cells build the tendon enthesis at the postnatal stage (5,8,9). Ablation of these cells resulted in decreased fibrocartilage formation and collagen disorganization. Conditional deletion of Smo (5,8,9). Ablation of these cells resulted in decreased fibrocartilage formation and collagen disorganization. Conditional deletion of Smo

RESULTS

Primary cilia are prevalent at the postnatal tendon enthesis

We previously demonstrated that Hh signaling is essential for tendon enthesis formation; furthermore, others have shown that primary cilia are critical for transduction of Hh signaling to musculoskeletal...
cell nuclei (5, 15). Therefore, we investigated tendon enthesis cell ciliogenesis during tendon enthesis development into maturity in a murine model. Antibodies against ciliary axoneme markers, including acetylated αtubulin, IFT88 (Intraflagellar transport protein 88) (ciliary axoneme markers), and/or the centrosome marker pericentrin, were used for immunostaining to detect primary cilia of supraspinatus tendon enthesis cells from different developmental stages (Fig. 1 and fig. S1). Cilium incidence (the number of ciliated cells normalized by the total cell number) of tendon enthesis cells increased markedly in the first 2 weeks postnatally, coincident with the pattern of Hh activation.

Fig. 1. Ciliogenesis of tendon enthesis cells during postnatal development coincides with Hh signaling and enthesis mineralization. (A to C) Immunofluorescence staining of Hh component Gli1 (gray) at the supraspinatus tendon enthesis (A) and primary cilia via acetylated tubulin (red, white arrowheads) at the enthesis (B) and midsubstance (C). Panels below (B) and (C) are magnified images corresponding to the colored rectangles. Arrowheads mark primary cilia; 4′,6-diamidino-2-phenylindole (DAP) stains nuclei; P1, postnatal day 1; W1, postnatal week 1. Scale bars, 10 μm (panel insets). (D and E) Safranin O staining of the tendon enthesis and midsubstance at different developmental stages. Dashed lines mark the tendon enthesis. (F) Illustration of the transduction of Hh signaling inside the primary cilium. IFT, intraflagellar transport protein. (G) Quantification of the incidence of ciliated cells at the tendon and tendon enthesis as well as Gli1-positive (Gli1+) cells, normalized by the total cell number. Four to five mice were analyzed per time point; all data are represented as means ± SD; n represents the number of cells counted. a, P < 0.05 compared to W0; b, P < 0.05 compared to W1; c, P < 0.05 compared to W2; d, P < 0.05 compared to W4; e, P < 0.05 compared to W6; f, P < 0.05 compared to W13.
[i.e., glioma-associated oncogene (Gli1) expression] (Fig. 3A, B, D, and G). In contrast, tendon midsubstance cells maintained a low level of ciliogenesis throughout postnatal development (Fig. 3E, F, and G). The proportion of tendon enthesis cells with cilia increased significantly between postnatal weeks 1 and 2 (W1 and W2), from 4.6 to 29.7%, and then decreased gradually to 12.1% by W13. Correspondingly, expression of Gli1 protein, the downstream effector of Hh signaling, changed in parallel with cell ciliogenesis during enthesesis development (Fig. 3H, I, and J). Enthesis cells had the highest activation of Gli1 and concurrent cilium incidence at W2 (Fig. 3G), coinciding with the onset of mineralization of the supraspinatus tendon enthesis (Fig. 3H) (16). These results imply that primary cilia contribute to Hh-driven and loading-regulated entheses mineralization.

There is an inverse relationship between in vivo loading and primary cilium incidence

Motivated by the mechanosensory role of cilia in chondrocytes and osteocytes (17–19), we evaluated the connection between primary cilium incidence and tendon enthesis mechanoresponsiveness during postnatal development. Tamoxifen (TA)–inducible GliCreERT2R.26Ros26mTomG mice were used to track Gli1-lineage (Gli1-lin) cells. Mice were injected with TA at P14 (TA14) or P28 (TA28) and sacrificed at P56. Shoulders were paralysed starting at birth using botulinum toxin A (BtxA) injection, a model that we previously established to unload the supraspinatus tendon enthesis and leads to defects in tendon enthesis formation (6). Compared to the enthesis from contralateral control shoulders, unloading increased cilium incidence by 33% in TA14 mice and 66% in TA28 mice, respectively (Fig. 3A and 3B). Similarly, unloading increased the Gli1-lin cell population by 48% in TA14 mice and 59% in TA28 mice (Fig. 3C). Unloading did not significantly change cilium incidence in the Gli1-lin cells in TA14 mice but resulted in a 31% decrease of cilium incidence in TA28 mice (Fig. 3D). However, cilium incidence of non–Gli1-lin cells was increased by 109 and 124% in TA14 and TA28 mice, respectively, after unloading (Fig. 3E). Unloading from birth through P56 led to significant up-regulation of genes related to ciliogenesis (e.g., IFT88, IFT80, and Dync2li1) and Hh signaling (i.e., Ptch1, Gli1, Gli2, and Gli3; Fig. 3F). These results demonstrate that biophysical forces drive ciliogenesis and activation of Hh signaling during entheses development.

To determine mechanoresponsiveness of cilia in the adult enthesis, BtxA was used to unload tendon entheses, and treadmill running was used to overload tendon entheses (7). Consistent with the effects of unloading during development, 2 weeks of unloading led to increases of 166% in cilium incidence and 175% in Gli1-positive (Gli1+) cells (Fig. 3A and 3E). Similarly, 4 weeks of unloading led to increases of 142% in cilium incidence and 128% in Gli1+ cells. In contrast to results during postnatal development, unloading of skeletal mature tendon entheses did not significantly change cilium incidence of Gli1+ cells, demonstrating that cilium assembly was not fully synchronized with Hh signaling in the adult tendon enthesis. In contrast to the effects of unloading, overloading led to a 48.7 and 56.8% decreases in cilium incidence after 2 and 4 weeks of treadmill running, respectively (Fig. 3I and 3J). Running-induced overloading did not modify the population of Gli1+ cells nor did it change cilium incidence of Gli1+ cells. Overloading through 4 weeks of treadmill running led to down-regulation of genes related to ciliogenesis (i.e., IFT88, KifA, and Dync2li1) and Hh signaling (i.e., Gli2; Fig. 3F). In summary, unloading and overloading experiments in postnatal and adult animals demonstrate an inverse relationship between loading and cilium incidence/Hh activation.

Primary cilia are necessary for entheses formation

The loading and unloading data demonstrate modulation of ciliogenesis at the tendon enthesis in response to in vivo loading. To extend our understanding of the role of primary cilia at the enthesis, we examined their necessity for entheses formation. Tendon-specific (Scx-Cre) conditional deletion of IFT88, a ciliary gene, was achieved using ScxCre;IFT88fl/fl (cKO) mice (20). cKO mice had a 64% decrease in cilium incidence compared to their littermate controls (Fig. 4A and 4B). Phenotype analysis from P10 to W13 demonstrated clear effects of cilia disruption on enthesis formation. The wild-type (WT) enthesis had rounder and apparently larger cells compared to cKO, consistently from P10 to W13 (Fig. 4C and 4D). This alteration in fibrocartilage cell phenotype was confirmed by changes in expression of collagen X, a marker for hypertrophic chondrocytes; collagen X expression was significantly decreased by 67% in enthesis from cKO mice compared to entheses from WT mice at 8 weeks (Fig. 4E, F, and G and fig. S3D). Cortical and trabecular bone were not affected by IFT88 knockout until W13 (Fig. 4H). At this time point, cKO mice had significantly deteriorated bone morphology of the humeral head, with thinner cortical bone and less mineralized enthesis fibrocartilage (Fig. 4I, J, and K and fig. S3D). Furthermore, the tendon enthesis from 13-week-old cKO mice had decreased structural properties (i.e., maximum force and stiffness) and increased material properties (i.e., stress and modulus; Fig. 4I), with drastically smaller cross-sectional areas in cKO tendon entheses (fig. S3A). IFT88 knockout in Scx-expressing cells led to overall changes in mouse physiology. The growth of cKO mice was slower, with significantly lower body weights compared to WT controls (fig. S3A and B). Thirty percent of cKO mice died between W6 and W13, possibly due to polycystic kidney disease directly caused by deletion of IFT88. Loss of IFT88 fibrocartilage cells resulted in altered mouse locomotion, e.g., swing speed and cadence (fig. S3A).

Ciliary Hh signaling mediates mature enthesis adaptation to in vivo loading

To examine the interaction between primary cilia, Hh signaling, and in vivo mechanical loading, we generated mice that harbored a tendon-specific loss of the Hh receptor Smo (Smosmoothed) and subjected them to unloading and overloading protocols (Fig. 4A). Deletion of Smo caused a 55.0% increase in tendon enthesis cells with primary cilia, indicating that cilia assembly was maintained or induced by Hh disruption (Fig. 4H and 4I). Overloading and unloading also induced cilia disassembly and assembly, respectively, in the enthesis from ScxCre;Smofl/fl cKO and WT mice, further demonstrating a mechanosensory role of cilia at the enthesis (Fig. 4D). Consistent with our previous study, loss of Smo in Scx-expressing cells led to a loss of enthesis fibrocartilage and decreased structural and material properties (Fig. 4I, J, and K and fig. S4A). When considering entheses mechanoresponsiveness, both overloading and unloading led to changes in enthesis mineralization (i.e., increased and decreased densities of cortical bone after overloading and unloading), bone morphometry, and mouse gait in WT mice (Fig. 4I and fig. S4). In contrast, Hh deletion mitigated many of these loading-induced changes in the tendon enthesis of WT mice: cross-sectional area in WT mice decreased after both overloading and unloading (Fig. 4I), stiffness and ultimate stress decreased after unloading, and ultimate stiffness and ultimate stress decreased after unloading.
stress increased after overloading; none of these outcomes were affected by loading in SccXCre;Smo<sup>fl/fl</sup> cKO mice (Fig. 3B). When examining the morphometry of bone adjacent to the tendon enthesis, overloading increased and unloading decreased cortical bone densities (tissue mineral density) and trabecular bone densities (bone mineral density) in both genotypes (Fig. 3C). Similar results were seen in other bone morphology outcomes (fig. S4B). At the whole animal level, both swing speed and cadence were increased by unloading for both genotypes (Fig. 3D and fig. S4C).

**DISCUSSION**

The current study demonstrated that tendon enthesis formation and function are driven by primary cilium-dependent biomechanical and Hh signaling. We first identified that the primary cilium is critical for tendon enthesis mechanoresponsiveness. Ciliogenesis during enthesis development and maturity was inversely driven by physiological loading. In addition, cilium incidence at the tendon enthesis increased during enthesis mineralization and was synchronized with Hh signaling, supporting the concept of interplay between Hh signaling and primary cilia (21). This is consistent with previous reports that both primary cilium and Hh components change dynamically during muscle differentiation (22). Further evidence of this interplay was shown through deletion of Hh signaling, which caused abnormal ciliogenesis and diminished mechanoresponsiveness at the tendon enthesis. Most markedly, the deletion of primary cilium from the tendon enthesis caused significant structural and compositional defects to the enthesis. Therefore, primary cilium are key regulators of enthesis formation and maintenance, modulating their assembly in response to biophysical forces and their interaction with Hh signaling.

Consistent with the current in vivo results, an inverse relationship has been demonstrated previously between ciliogenesis and mechanical loading in zebrafish (17, 23, 24). Mechanical stimulation above a threshold leads to cilia shortening and disassembly, and removal of mechanical loading leads to cilia elongation and assembly (24–26). This response may be an effort of cilia to scale their sensitivity according to the loading signal. Cilia elongation or assembly increases the mechanosensitivity of the cilium or increases the number of mechanosensors on the ciliary membrane, respectively, which improves the cell’s ability to sense mechanical cues from its environment.