U·P·O·J

Morphogenesis of Fibrous Structures in the Embryonic Knee is Severely Disrupted by a Lack of Muscle Contraction

Tonia K. Tsinman[1]
Saima Ahmed[2]
Aurélie Levillain[2]
Yuming Huang[2]
Niamh C. Nowlan[2]
Nathaniel A. Dyment[1]
Robert L. Mauck[1]

[1]University of Pennsylvania
Philadelphia, PA

[2]Imperial College of London
London, UK

Disclosures

T Tsinman (N), S Ahmed (N), A Levillain (N), Y Huang (N), N Nowlan (N), N Dyment (N), R Mauck (8, JOR Spine)

Introduction

Precise tissue morphology and positioning within the knee are critical for the patellar tendon, cruciate ligaments, and menisci to mechanically support joint function. Shape and organization of these knee fibrous elements is coordinately established during embryonic joint formation, whereby joint cells are specified and patterned within the hindlimb cartilage anlagen, and become distinct structures following joint cavitation[1,2]. Contraction of skeletal muscle is a key regulator of the joint morphogenesis process[3]. In particular, Splotch-delayed (*Spd*) and Muscular Dysgenesis (*mdg*) mouse models, in which skeletal muscle fails to develop (*Spd*), or is non-contractile (*mdg*), exhibit joint cavitation failure, resulting in fusion of most articulating joints[4]. Surprisingly, aside from patellar fusion, few overt changes have been reported in the knee joints of these mice, leading to the belief that muscle contraction does not significantly contribute to knee development[4,5]. This notion, however, is confounded by chick studies showing meniscus dissociation and molecular signaling alterations following embryonic hindlimb paralysis[6,7]. To date, however, no study using the available murine models has assessed how absence of muscle contraction impacts the fibrous tissues unique to the knee joint. Here, we demonstrate that deficiency in muscle loading in mice causes aberrant changes in the establishment of fibrous tissues of the developing knee that far exceed those previously reported[4,5].

Methods

mdg mutant mouse embryos were harvested alongside littermate wild-type (WT) controls at Thieler Stage (TS) 24 and 27, along with TS27 *Spd* mutant embryos and littermate WT controls. Knee joints from three animals per time point and genotype were dissected, fixed in formalin, cryo-embedded, and serially cryosectioned (8-16μm) in the sagittal and/or coronal planes across the width of the joint. Sections were permeabilized (0.1% Triton-X), stained with Alexa-Fluor Phalloidin 546 (actin), and counterstained with Hoechst-33342 (nuclei). Imaging was done by laser-scanning confocal. Second harmonic generation (SHG) for visualization of fibrillar collagen was performed by multiphoton microscopy (20X objective). For nuclear aspect ratio (NAR), z-stacks (60X, 0.2μm step-size) for individual nuclei were acquired (from PT sections of two TS24 animals/genotype), and aspect ratios were calculated from maximum intensity projections using Fiji. Measurements were pooled for WT/mdg samples and compared via a Mann-Whitney U Test ($p < 0.05$ cutoff).

Results

Knee joints of TS27 *mdg* (non-contractile muscle) mutants contained patellar tendon (PT), ACL, and PCL structures, though all were greatly reduced in thickness (Figure 1a; Figure 1b (PT)). Likewise, SHG imaging showed aligned collagen fibers in the *mdg* PT and cruciate tissues that were diminished compared to WT controls at this time point (Figure 1c). Strikingly, an extra tissue structure–aligned parallel to the PT and with femoral and tibial attachments anterior to the ACL/ PCL was observed in all TS27 *mdg* mutants (Figure 1a, red arrowhead). This element appeared to be a fibrous ectopic ligament, as it contained both linearly aligned cells and collagen fibrils, similar to the adjacent tendon/ligaments (Figure 1b, c, "Ect. Lig.").

Additionally, all TS27 *mdg* knees had a complete absence of the medial meniscus anterior horn (Fig. 1b), a severe reduction in the medial meniscus body/posterior horn, and the majority of the lateral meniscus was missing (data not shown). These phenotypes were confirmed in the sagittal and coronal planes, and were also observed in TS27 *Spd* mice (data not shown). The vertical "ectopic ligament" and the missing anterior medial meniscus horn were already noted in *mdg* joints at TS24, around the time the knee joints cavitates (Figure 2a, b). However, at TS24, cells were properly positioned at the sites of the PT and cruciates in *mdg* samples (Figure 2a). While these resident cells exhibited linear alignment and fibrillar actin networks that

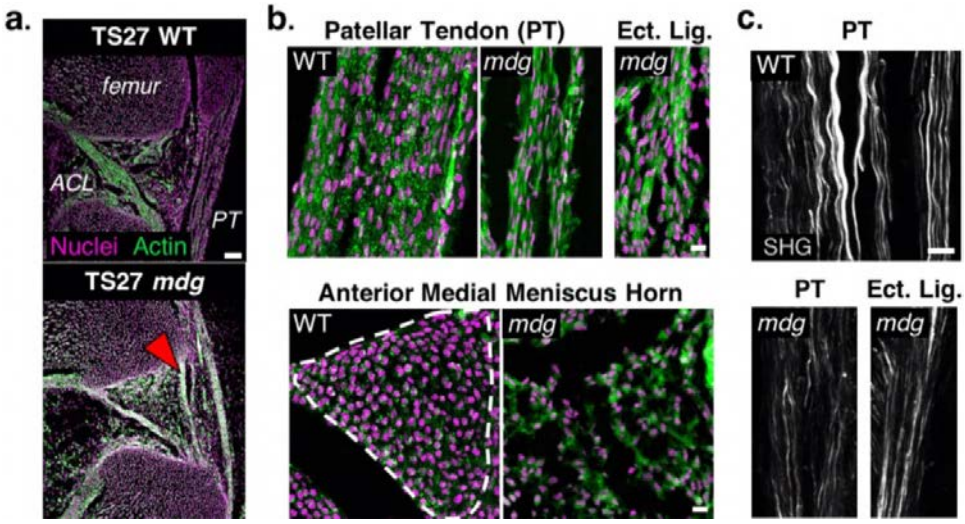

Figure 1. TS27 analysis. (A) Wild-type (WT) and *mdg* mutant sagittal knee sections stained for cell nuclei (magenta) and actin (green).Red arrowhead denotes an ectopic ligament structure ("ect. lig.") seen in the *mdg* joints. **(B)** Magnified views of WT and *mdg* indicated issues from sagittal serial sections.**(C)** Aligned collagen visualization by SHG (second harmonic generation) in the WT and *mdg* PTs and the *mdg*"ectopic ligament". SBs: 100μm **(A)**; 10μm **(B,C)**.

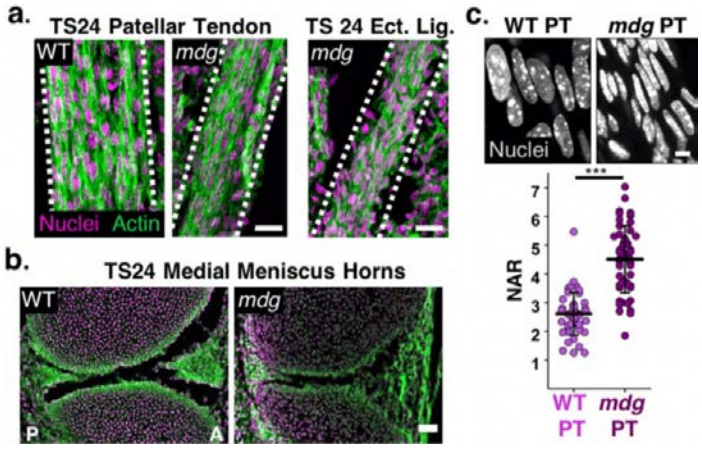

Figure 2. TS24 analysis. Sagittal views of cell organization in WT and *mdg* PTs and the *mdg* ectopic ligament ("ect lig.") **(A)** and medial meniscus horns **(B)** .A: anterior, P: posterior. **(C)** Nuclear aspect ratio (NAR) measures (n = 50/group, mean ± s.d) for nuclei of WT and *mdg* PT resident cells. ***: $p < 0.001$. SBs: 20μm **(A,B)**; 5 μm **(C)**.

resembled WT cells, their nuclei were significantly elongated (Figure 2c).

Discussion

This study demonstrates a varied, but overall profound, impact of muscle contraction on developing fibrous knee joint tissues. By the joint cavitation stage (TS24) in *mdg* mutants, cellular condensations were absent in the anterior regions of the medial meniscus (Figure 1b, Figure 2b) and throughout the lateral meniscus (data not presented), suggesting that defects in meniscus cell patterning may be responsible for the absence of these structures by TS27. Interestingly, meniscus morphogenesis phenotypes were asymmetric—with differential effects seen based on anterior-to-posterior and medial-to-lateral position. This highlights that location-specific biophysical and/or molecular cues may be guiding meniscus formation. Conversely, cells of the PT and cruciate ligament of *mdg* mice appropriately assembled by TS24 (Figure 2a) and were able to generate a fibrillar collagen network by TS27 (Figure 1c), suggesting that specification of the PT and cruciates initiates normally, despite abnormal muscle forces. However, the hyper-elongation of cell nuclei in the PT at TS24 (Figure 2c) points to an alteration in the mechanical microenvironment, perhaps due to increased resident cell contractility or a heightened level of tissue pre-stress. Either factor could contribute to the observed reduction in size of the PT and cruciates at TS27, through increased tissue micro-damage, cell shearing, or potentially cell death. Intriguingly, morphogenesis of an ectopic tissue consistently occurred when muscle was absent or non-contractile, with a defined position within the joint, and ligamentous tissue properties (Figure 1b, 2b). This finding demonstrates that cues downstream of a loss of muscle contraction may enable alternative cell condensations to persist; a mechanism that requires further investigation.

Significance

We demonstrate that, contrary to the established understanding, muscle contraction is critical for murine knee joint development, and acts to direct the proper morphogenesis of the PT, menisci, and cruciate fibrous tissues, rather than impacting knee joint cavitation.

Acknowledgements

This work was supported by ERC Grant #336306 and the NIH (R01 AR075418 and R00 AR067283).

References

1. **Shwartz+,** 2016
2. **Gamer+,** 2017
3. **Nowlan+,** 2010
4. **Kahn+,** 2009
5. **Eyal+,** 2015
6. **Mikic+,** 2000
7. **Roddy+,** 2011.